# THE ROLE OF MATRIX METALLOPROTEINASES IN MURINE FACIAL MORPHOGENESIS

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1998

## ACKNOWLEDGMENTS

I would like to thank many people that helped me along the way during these five years of graduate school. Thanks go to my mentors, Dr. Michael Humphreys-Beher and Dr. Linda Brinkley, who encouraged me to think on my own. I value their advice and guidance and hope that we will continue our friendship for years to come.

To all members of my committee, Dr. Nancy Denslow, Dr. Greg Schultz, Dr. David Muir and Dr. William Williams, thanks go for the support and belief that I could finish this project. Their expertise and advice helped me tremendously.

A few people were essential for my acceptance to graduate school, Dr. Mary Jo Koroly, Dr. Sheldon Schuster and Dr. Arnold Bleiweis. My first year tuition was covered by the ICBR while I worked in the Protein Core Facility. My gratitude goes to all of them. I can not forget to thank everybody in the Protein Core Facility where I worked for almost 2 years and where I learned my baby steps in molecular biology. Special thanks go to Dr. Denslow, Ms. Marjorie Chow and Mr. Hung Nguyen. My research and stipend were covered in part by my mentors' NIH grants and by the University of Florida. Other institutions also responsible were the Florida/Brazil Institute and the Hispanic Dental Association.

I would like to thank past and present oral biology students that gave me their support and made these years in graduate school enjoyable, Dr. Heather Allison, Mr.

Jason Brayer, Dr. Lina Bueno, Dr. Dong Mei Gao, Dr. James Kohler, Dr. Shawn Macauley, Dr. Greg Oxford, Dr. Chris Robinson, Ms. Lori Wojciechowski. I can not forget to thank the MHB lab where working is a fun process. Special thanks go to Mr. Micah Kerr for assistance with the PCR experiments, Mrs. Amy Shawley for assistance with the culture experiments and Ms. Jennifer Lowry for her assistance. In the Brinkley lab, special thanks go to Dr. Yan Du for her assistance with the zymography experiments.

My eternal gratitude goes to Drs. David and Nancy Denslow for all the support they gave us through these years. Their friendship and caring are beyond words. To all my family at home, my parents Antonio and Antonia Tavares da Costa, my sister, Ana Maria, and my brother, Antonio Jose, and family, to Dr. Jose Dantas and Valniza da Silveira, Dr. Polina da Silveira and Dr. Romulo da Silveira and family, thanks for the support and love.

This dissertation is dedicated to my husband, Dimitrio Sergio da Silveira, who never let me give up and always encouraged me with unconditional love and support. This could not be accomplished without him and the endless hours of his help.

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Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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December 1998

Chairman: Michael Humphreys-Beher
Major Department: Oral Biology

Facial morphogenesis is dependent on extracellular matrix (ECM) synthesis and accumulation. It is tightly regulated by the interaction of the proteins that degrade the extracellular matrix in a given time and space. Current literature suggests that matrix-degrading enzymes play a role in many developmental processes. We suggest that facial development is one of these processes. Molecules known or expected to be involved in various aspects of normal development in animal models have been tested in humans with defects and have been shown to be involved in a number of common malformations. This study allows determination of whether matrix proteinases play a role in primary palate and mid-face morphogenesis.

The objectives of this study were to detect changes in the expression of ECM and matrix metalloproteinases (MMPs), to measure the amount of ECM message being

produced at the time of facial formation and to determine the role of MMPs in facial development by perturbing their function *in vitro*. Immunoblots demonstrated the presence of basement membrane ECM components and MMP-2, MMP-3 and MMP-9 in the facial processes. Quantitative RT-PCR was used in order to measure the production of ECM molecules' mRNA and indicated an increase in the number of these molecules as development progresses. Specific MMP inhibitors were used to block proteinase function in cultured embryo heads. After 7 days in culture, controls demonstrated complete closure whereas experiments showed a cleft resultant from the lack of fusion between the lateral and medial nasal processes. These data suggest that production of ECM mRNA and degradation of ECM by MMPs are occurring in the facial processes at the time of facial development in the mouse and that the MMPs are involved in the formation of the mid-face.

Our study may provide insights into normal and abnormal facial development particularly relevant to the occurrence of cleft lip. By identifying the players and providing indications on how these players alter development, we can have a better understanding of the whole process. This may lead to strategies for preventing the action of perturbing agents and compensating for defective genes.

# INTRODUCTION

The goal of this research is to achieve a better understanding of the role of the extracellular matrix (ECM) and matrix-degrading enzymes in normal mid-face and primary palate morphogenesis. Many morphogenetic processes require balancing the synthesis and destruction of ECM. These changes can be mediated by regulating the synthesis of ECM components, matrix-degrading enzymes, and their inhibitors, by differential proteinase-mediated degradation of ECM, or both. A vast array of factors, including growth factors, cytokines, hormones, steroids, vitamins, protooncogenes and even the molecules themselves are known to regulate the transcription of ECM, proteinase and proteinase inhibitor genes (Pan et al., 1995; Gutman and Wasylyk, 1990; Wasylyk et al., 1991; Campbell et al., 1991; Lin, Georgescu and Evans, 1993). Less work has been done on the interaction of matrix-degrading enzymes and their substrates as it relates to specific morphogenetic events. The present proposal will focus on just such activities in the development of the mid-face and primary palate.

Several studies have demonstrated the presence of several ECM molecules, matrix metalloproteinases (MMPs), plasminogen activators (PAs), tissue inhibitors of matrix metalloproteinases (TIMPs) and their messages in the facial processes over the course of murine facial development (Chin and Werb, 1997; Iamaroon and Diewert, 1996; Iamaroon et al., 1996). The combined activation of various MMPs is essential for the efficient turnover of the structure, and the expression of MMP genes is regulated for

this purpose. This regulation is complex and can occur at different levels: intracellularly by transcriptional and translational regulators, extracellularly by proteolytic activation of the proenzyme and binding of inhibitors.

The following hypothesis was proposed for this study: *Formation of the normal primary palate and the mid-face is dependent on the morphogenetic movements of the facial processes caused by specific changes in tissue architecture. These changes involve temporo-spatially localized alterations in the distribution of the ECM molecules. Proteinase mediated degradation of the ECM molecules plays a role in these changes.*

*Also, correct facial and upper lip formation depends on the degradation of the basement membrane of the facial processes by proteases.*

From the above hypothesis, we can predict that

1) The presence of matrix metalloproteinases in the facial processes will be associated with the changes in the distribution of their target ECM molecules during remodeling.

2) Disrupting the normal temporal and spatial sequence of appearance of matrix degrading proteinases will result in abnormal facial morphogenesis.

## Specific Aims

Aim 1 is to describe the temporal appearance of MMPs, their target ECM molecules and their messages during mid-face and primary palate morphogenesis *in vivo*.

Aim 2 is to determine the effects of altering the expression of MMPs by inhibiting the matrix-degrading proteinases on primary palate and mid-face morphogenesis *in vitro*.

# LITERATURE REVIEW

## Introduction

One of the major determinants of the development and maintenance of three-dimensional form in animals is the assemblage of the extracellular matrix (ECM) (Hay, 1981). In the embryo, the ECM is the scaffolding that helps determine tissue patterns. In the adult, it serves to stabilize these same patterns. Extracellular matrix can be found as both interstitial matrix or organized as a basement membrane interposed between tissue layers. In early development, interstitial matrix is found between cells in the mesoderm and a basement membrane is found between ectoderm or endoderm and the underlying mesoderm. As development proceeds and differentiation takes place, interstitial matrix is found in connective tissues and as a basement membrane underlying epithelial or endothelial layers, separating them from the underlying connective tissue, as well as around muscle and nerve cells.

Collagens, proteoglycans, glycosaminoglycans (GAGs) and glycoproteins compose the extracellular matrix. There are at least sixteen different types of collagen described to date. Their composition differs in the amount and type of the basic unit of collagens, the triple helix (comprised of three polypeptide $\alpha$ chains) and also in their configuration and tissue location.

The proteoglycans are a diverse family of molecules distinguished by a core protein attached by one or more GAG side chains. Recent review of the nomenclature of the proteoglycans classifies this class of glycoproteins according to their function or their protein core whereas in the past, it reflected the chemical composition of the GAG chains (Ayad et al., 1994). The glycosaminoglycans are a group of carbohydrates that include chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate (HS), keratan sulfate (KS) and hyaluronan (HA).

Fibronectin, vitronectin and laminin are examples of glycoproteins found in the extracellular matrix. Similar structure and function are attributed to this class of proteins. Many hold RGD sequence motifs that mediate cellular adhesion. They have been implicated to influence cell behavior by allowing attachment and migration and to have the ability to concentrate ions and growth factors.

The interstitial ECM is composed of various collagens, glycosaminoglycans, glycoproteins and elastin bathed by a tissue fluid. In the adult, it is the substrate on which fibroblasts and macrophages normally reside. The basement membrane, defined originally by light microscopy, is composed of substructures visible only by electron microscopy, the principal one being the basal lamina. The basal lamina is a thin sheet-like structure composed mostly of glycosaminoglycans, collagen and proteoglycans.

During morphogenesis tissues must change size, shape and character. This requires spatial and temporal changes in the distribution and composition of both the interstitial and the basement membrane ECM. Such changes have been shown to take place during the development of several embryonic structures (Fitch, Mayne and Linsenmayer, 1983; Bernfield et al., 1984; Thesleff et al., 1981; Sahlberg et al., 1992).

The synthesis and degradation of ECM is integral to morphogenesis (Nakanishi et al., 1986). Tissue development and structure is controlled by dynamic and interactive relationships between cells and the ECM they secrete. The development of the face and primary palate is no exception. Yet little work has been done on the temporal and spatial changes in the ECM during the development of these important structures.

## Description of Facial Formation

The development of the face may be considered to begin at the time of neural tube formation with the production of a cell population crucial to facial development, the neural crest cells. These cells originate from the neural tube and migrate extensively in the subectodermal region of the loose mesenchyme to their final destinations throughout the embryo. There they give rise to a number of differentiated cell types. The neural crest cells will ultimately give rise to most of the differentiated structures of the face and neck since they form the mesenchyme of the swellings that will give rise to these structures, the frontonasal process and the paired branchial arches. The facial skeleton, connective tissues, parts of the teeth and nerve fibers are all of neural crest origin.

The formation of the five primordia that will give rise to the structures of the face and neck begins about the fourth week of development in humans or about gestational day eight in mice. These primordia are the frontonasal process, and the paired maxillary and mandibular processes. The frontonasal process is initially identifiable as a single swelling overlying the forebrain. At about the same time, paired swellings develop on either side of the developing pharynx, the first branchial. Subsequently four other paired swellings will develop (branchial or pharyngeal arches 2-5) although branchial arches 4

and 5 are rudimentary and very small. The first or mandibular arches rapidly subdivide into superiorly located maxillary processes and an inferiorly located mandibular process. These branchial arches are composed of neural crest-derived mesenchyme, sometimes called ectomesenchyme, encased by a layer of ectoderm (Richman, Rowe and Brickwell, 1991). Extracellular matrix is present between these mesenchymal cells and in the basement membrane between the mesenchyme and the overlying ectoderm.

The frontonasal process will also subdivide into a medial nasal and a pair of lateral nasal processes. A critical stage in this morphogenetic event is the elevation and outgrowth of the nasal processes around the nasal (olfactory) placodes. The nasal region is first identified by two thickened epithelial regions (nasal placodes) that curl to form a nasal groove that delineates the lateral from the medial nasal process on each side of the future nose. The enlargement and fusion of the medial nasal and lateral processes (derived from the frontonasal process) with each other and with the maxillary process (derived from the first branchial arch) will determine the following structures: the nose, the upper lip and the primary palate, which will ultimately constitute the middle third of the face (Johnston and Bronsky, 1995).

Facial development occurs over about an eight-day period in humans and over a four-day period in mice. Major changes in spatial relations take place during this time: the brain enlarges, the distance between the nasal placodes narrows, the frontonasal process elongates vertically and narrows, and the maxillary processes from both sides grow forward and enlarge (Diewert and Shiota, 1990; Diewert and Lozanoff, 1993a, 1993b; Diewert, Lozanoff and Choy, 1993; Rudé et al., 1994). It has been demonstrated that the facial processes have different growth patterns and directions in different regions

of the face that may contribute to changes in morphology (Diewert, Wang and Tai, 1993). As facial development continues, the lateral and medial nasal processes on both sides of the developing nose make contact with each other and with their respective maxillary process (fig.1). Epithelial adhesion and later fusion occur at the inferior part of the nasal groove. Subsequently this nasal fin disappears as adhered epithelial cells of the apposed lateral and medial nasal processes die or transdifferentiate into mesenchymal cells, basal lamina is degraded and a mesenchymal bridge between the processes that allows tissue continuity is formed (Diewert and Shiota, 1990).

## Models for Studying Facial Morphogenesis: Chicken and Mouse

Studies of morphogenesis require the use of experimental models. Early studies of facial development were confined to chick embryos. Chick development occurs in a large egg that develops outside the uterus that can be windowed to allow direct surgical manipulation of the large embryo. Although some differences were observed between avian and mammalian craniofacial development, researchers in the field often chose to continue their work using the chicken model, because of its accessibility. With the discovery that quail cells were easily histologically distinguished from chick cells, tissue recombinations and chimera studies in culture were exploited to study craniofacial development. The advent of new techniques such as antibodies and whole embryo culture enabled the re-introduction of the mammalian embryo as a model for studies of craniofacial development.

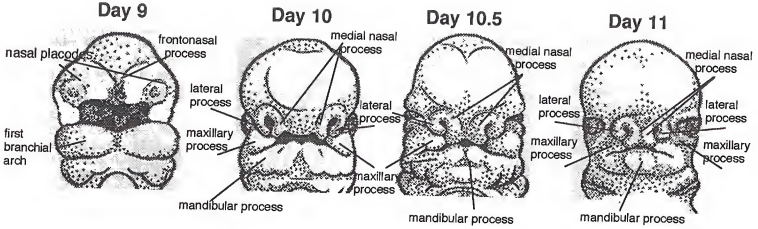


Fig 1. Mouse facial development from gestational days 9 through 11. At day 9, the nasal placodes and the first branchial arch are easily recognized. At day 10, the frontonasal process divides into medial nasal process and lateral process. The first branchial arch divides into mandibular and maxillary process. At day 10.5, the facial process grow towards the mid-line, and at day 11, just prior to fusion, the lateral, maxillary process from each side of the developing nose make contact with each other and with the medial nasal process (modified from Mayer and Swanker, 1958).

Many transplantation and extirpation experiments in mammalian embryos have demonstrated that cells derived from the neural crest participate in the formation of the midfacial and visceral arch skeletal structures (Morris-Kay and Tam, 1987). Migratory pathways have been well defined by the application of antibodies and have shown that cells derived from different facial processes have different origins (Noden, 1988). Cells destined for the frontonasal and maxillary processes remain in close proximity to the prosencephalon while others move away from the neural epithelium (Johnston, 1966; Noden, 1975).

## Mechanisms of Face Morphogenesis: Cell Proliferation, ECM Synthesis and Degradation

### Cell Proliferation

It has been proposed that growth of the facial processes is due to the proliferation of the mesenchymal cells (Andersen and Matthiessen, 1967). Various studies have demonstrated that cells are proliferating at high rates in the mesenchyme of facial processes during facial development (Wilson and Hendrickx, 1977; Minkoff and Kuntz, 1977, 1978; Figueroa and Pratt, 1979; Gaare and Langman, 1980; Igawa et al., 1986; Minkoff, 1980, 1991). Additional research has found differences in mitotic activity within different facial regions. Growth rates were higher in the subepithelial mesenchyme of the maxillary processes than they were more interiorly (Bailey, Minkoff and Koch, 1988). Tissue recombination studies have demonstrated that the mesenchyme is dependent on the epithelium for its growth and survival (Saber and Minkoff, 1991; Richman and Tickle, 1989). Other studies have found similar increased cell proliferation

when different regions within the lateral and medial nasal mesenchyme were examined (Gui, Osumi-Yamashita and Eto, 1993). When the epithelium was analyzed, lower synthesis was found in fusing epithelia compared to non fusing areas as development progresses, supporting the idea that these cells are undergoing changes in preparation for contact and cell death (Kosaka and Eto, 1986).

Failure of fusion of the facial processes has often been attributed to defective outgrowth of the facial processes. In some instances, the processes are too small or are not properly positioned to permit contact and fusion to occur. Defective process formation has typically been attributed to an insufficient number of mesenchymal cells in the processes, a condition that might result from interference with neural crest cell migration to the area, decreased mesenchymal cell proliferation, or aberrant cell death (Noden, 1975; Minkoff and Kuntz, 1978). The excision of the frontonasal process in the beginning of facial morphogenesis in chick embryos results in arrested development of the upper beak and inhibited growth of the maxillary processes (McCann, Owens and Wilson, 1991). Similar operations involving removal of each facial process in cultured rat embryos demonstrated that removal of the medial nasal process results in a large number of malformations of the upper lip (Ohbayashi and Eto, 1986). The excision of maxillary and lateral processes, however, only resulted in a small number of malformations. The results can be interpreted in two ways. First, it has been demonstrated that the growth of the medial nasal process is greater than of the lateral process (Patterson and Minkoff, 1985) thus, the medial nasal process could extend to the maxillary process and substitute for the tissue lost. Second, since the medial nasal process is the biggest facial process, it

is likely the embryo cannot regenerate such a big tissue volume and malformations can result.

**ECM Synthesis**

It has also been suggested that synthesis of ECM plays a role in the facial process outgrowth (Sadler, Langman and Burk, 1980). Various studies have reported the incorporation of radiolabeled glucosamine into new glycosaminoglycan (GAG) molecules synthesized at the time of facial processes formation (Burk, 1983). The results demonstrate that over half of the GAGs synthesized during the period of mouse facial process formation and outgrowth are of hyaluronan (HA) form. Lesser amounts of chondroitin sulfate (CS), heparan sulfate and others are also synthesized.

The composition of the extracellular matrix of the facial processes has been studied by histochemical techniques, specific for visualization of ECM molecules (for example, tannic acid) and more recently by employing antibodies. Antibodies permit a better identification of the ECM components whereas specific staining procedures only facilitate speculation on the true identity of these molecules. For example, tannic acid (TA) may not preserve all the ECM. Collagen like fibers are seen without special preparation and are most prevalent in subepithelial spaces associated with basal lamina (Hall and MacSween, 1984), whereas fixation with TA enhances the retention of some other matrix components. Chondroitin sulfate, chondroitinase ABC and HA were all identified by different staining techniques and/or digestion with a specific enzymes as being components of ECM in the facial processes.

An immunofluorescent study of the composition of murine day 11 mandibular process mesenchyme described the presence of fibronectin, type II collagen and cartilage

specific proteoglycan (Richman and Diewert, 1987). Type I collagen was absent on early days 10 and 11, and its synthesis was correlated with chondrogenesis. The distribution of type IV collagen, laminin and fibronectin and their alteration has also been examined in the maxillary and medial nasal processes of chick embryos (Xu, Parker and Minkoff, 1990a). Differences in concentration of type IV collagen were noted within the basement membrane of the maxillary process and its localization was more prominent in the medial nasal basement membrane than in any other region. In the medial nasal process, type IV collagen appeared to be predominantly located in the basement membrane in the lateral regions, whereas in the central region, it appeared to be located in the epithelial cells. These differences were confirmed using two other monoclonal antibodies to type IV collagen. Laminin was found to be more evenly distributed in the basement membrane of the facial processes as was fibronectin within the mesenchyme. The results suggest that, during facial morphogenesis, primordia such as the maxillary processes, which undergo rapid changes in form, have alterations in collagen distribution to a much greater extent than laminin or fibronectin, which could serve as structural support for the basement membrane and the mesenchyme, respectively.

Synthesis of different ECM molecules in the basement membrane has also been implicated as influential in the epithelial-mesenchymal interactions of the maxillary processes (Xu, Parker and Minkoff, 1990b). In order to investigate whether the presence of specific basal lamina components was a requirement for epithelial-mesenchymal interactions, a study was conducted in which fluorescent monoclonal antibodies to laminin (Ln) and type IV collagen were employed to detect the presence of these components during tissue isolation procedures. Each isolated chick embryo epithelial and

mesenchymal portions of maxillary process produced different basement membrane components in tissue culture. Only laminin was found in isolated epithelia after 24 hrs in culture. When recombined, however, not only was immediate production of laminin observed, but the basement membrane was reconstituted by 24 hours *in vitro*, including type IV collagen, laminin and fibronectin. Therefore, the recombination of the epithelium with the mesenchyme altered the patterns of synthesis of ECM molecules. Since the examiners only looked for laminin and collagen IV, it is not known whether GAG components of basement membrane were present or being synthesized. They suggested that the alteration of synthesis of ECM molecules after tissue recombination can provide an environment for the generation of signals between the epithelium and the mesenchyme which are necessary for mesenchymal growth and survival, for example the passage of growth factors. Alterations of ECM composition of basal lamina can also be obtained by specific proteinase degradation.

In summary, the composition of the extracellular matrix of the facial processes has not been completely defined. Two different experimental models have been used, the chicken and the mouse and it is not known if their matrix compositions are the same. Overall, the mesenchyme of the facial processes appears to be composed primarily of glycosaminoglycan molecules, especially hyaluronan, but fibronectin and collagens are also present. The basement membrane contains type II (mandibular process) collagen, type IV collagen and laminin in different amounts, dependent on the region or process analyzed.

**ECM Degradation**

The morphogenesis of tissue and organs during development requires dynamic changes in the extracellular matrix composition. Extracellular matrix is present between mesenchymal cells and in the basal lamina underneath the epithelium layer. This morphogenesis can be achieved by differential synthesis or by degradation or both. The rapid changes in tissue form that occur during morphogenesis suggest that both the interstitial matrix and the basement membrane must be remodeling. The basement membrane also acts as a medium by which inductive epithelial-mesenchymal interactions are mediated and is also associated with directed cell migration. Both of these processes require the ability to locally, quickly and specifically alter the composition of the basement membrane.

The difference in staining of collagen IV observed in previous studies (Xu, Parker and Minkoff, 1990a) suggests that basement membrane degradation occurs in regions of outgrowth of the maxillary process, more specifically away from the fusion regions. These patterns may be associated with the morphogenetic events of primary palate formation. It is known that basement membranes show altered temporo-spatial patterns of distribution of components such as laminin, fibronectin, and collagens IV and I during organogenesis. Such changes have been reported during ocular development (Fitch, Mayne and Linsenmayer, 1983), salivary gland morphogenesis (Bernfield et al., 1984) and tooth development (Thesleff et al., 1981; Sahlberg et al., 1992). In facial morphogenesis, degradation of the basement membrane is a necessary step in mesenchymal bridge formation and process fusion. The distribution of basement membrane in the mesenchymal bridge of mouse primary palate was described by using

fluorescent antibodies to laminin, type IV collagen and fibronectin (Iamaroon, Tse and Diewert, 1996).

More recently, Iamaroon and Diewert (1996) examined the distribution of basement membrane components in the mouse primary palate by immunofluorescence. They report the presence of laminin, fibronectin and collagen IV in the basement membrane that becomes fragmented along the epithelial seam prior to fusion. This is suggestive of a rapid disruption of the basement membrane in association with formation of a mesenchymal bridge. Fibronectin was also found in the mesenchyme of the facial processes. After fusion or closure, basement membrane components are found intact along the margins of the facial processes. The continuance of the existence of the epithelial seam and the basement membrane may be associated with cleft lip formation (Diewert and Wang, 1992). Similar breakdown of basement membrane and regression of the epithelial components can be found in other embryonic systems, such as during the development of the tooth, involution of mammary glands and development of secondary palate (Thesleff, Partanen and Vainio, 1991; Dickson and Warburton, 1992; Diewert and Wang, 1992; Morris-Wiman and Brinkley, 1993).

Matrix degradation appears to play a role in facial morphogenesis in two ways: interstitial and basement membrane extracellular matrix composition is altered for the necessary shape changes of the lateral process and subsequent curling of the nasal groove; and turnover of the basement membrane is associated with expansion and growth of the facial processes and at the time of fusion, its degradation allows mesenchymal continuity.

The morphogenic movements of the lateral process that lead to the curling of the nasal groove require alterations in tissue architecture. The alterations result from temporo-spatial changes in the distribution of the ECM in the interstitial matrix and in the basement membrane between the mesenchyme and the epithelium of the processes. Also, by specific temporo-spatial changes in the interstitial matrix and basement membrane composition, differential communication could take place between the epithelium and the mesenchyme of the facial processes. This could direct the growth of the facial processes, such as the changes in ECM composition in reorientation of palatal shelves in culture (Morris-Wiman and Brinkley, 1993), and act as a stimulus for cell differentiation or death. At the same time, the basement membrane must be degraded in regions of the facial processes where growth is constant or intensified or where processes fuse to allow mesenchymal continuity to take place.

Little work on the role of matrix-degrading enzymes in facial morphogenesis has been done to date. Some matrix metalloproteinases and their inhibitors have been localized in the craniofacial complex (Kinoh et al., 1996; Iamaroon et al., 1996, Chin and Werb, 1997). Among them, MMP-2 seems to be constitutively expressed during embryogenesis. Matrix metalloproteinase-2 has been localized by immunohistochemistry in the tips and the periphery of the nasal processes, lateral and medial nasal, at the time of growth and just prior to fusion (Iamaroon et al., 1996). After fusion, staining was significantly reduced with some remaining at the peripheral regions of the formed nostril.

Chin and Werb (1997) also demonstrated expression of MMPs and TIMPs during mandibular development in culture. When these MMPs activities were blocked by specific inhibitors of matrix metalloproteinases, fusion of mandibular processes and

tongue formation were arrested or delayed in a dose dependent manner (Chin and Werb, 1997). This study also showed abundant expression of MMP-2 by gestational day 9 in the first branchial arch in the stroma underlying the epithelium, making MMP-2 the most likely candidate for the major player in mandibular development. Although inhibition with synthetic broad-spectrum inhibitors of matrix metalloproteinases was successful, attempts of blocking specific MMP activity by antisense oligonucleotides failed.

Therefore, it is understood that identification of the temporal and spatial distribution of the major matrix molecules and their proteinases is an important step in understanding facial morphogenesis, and an integral part of this study.

## Experimental Alterations of ECM in Facial Processes

It has previously been shown that perturbation of some ECM molecules (glycosaminoglycans and collagen) results in the production of cleft palate (Brinkley and Vickerman, 1982). Proper temporo-spatial distribution of some ECM molecules in mammalian embryonic palatal tissue is required for normal development of the secondary palate. The alterations of these patterns of molecular distribution are not attributable to specific synthesis of the molecules (Morris-Wiman and Brinkley, 1992) and likely involve specific, local ECM degradation. It is hypothesized that the same could be true for facial development.

Several studies have utilized agents that degrade ECM or interfere with their synthesis. Hyaluronic acid has been shown to be a major constituent of the facial processes which will give rise to the primary palate (Burk, 1983). Streptomyces hyaluronidase specifically degrades HA and was injected into mouse embryos that were

cultured up to 24 hrs (Burk, 1985). The results of these studies were inconclusive since after 8 hrs of treatment, circulation in cultured embryos ceased and cellular degeneration followed.

Other studies have used various inhibitors. Median facial clefts were induced in mice by using DON (Diazo-Oxo-Norleucine), a glutamine antagonist that affects glycosylation, mesenchymal proliferation and synthesis of extracellular matrix components (Burk and Sadler, 1983), specifically glycosaminoglycans and proteoglycans. A decrease in cell density was observed in all areas of facial mesenchyme after treatment with DON as measured by $^{3}H$ thymidine incorporation. However, these results were inconclusive because of the dose of the compound and its potential toxicity. The observed decrease in cell density was probably associated with cell death. Other experiments reported a reduction in glycoprotein and GAG synthesis when DON was administered in cultured mouse secondary palatal shelves (Greene and Pratt, 1980). However, the appropriate control, which would have been the addition of glutamine or glucosamine to the culture medium to override the inhibitory effect, was not performed.

A fair number of teratological studies have indicated the medial nasal process as being the main site of action of various teratogens during facial development. Retinoic acid (RA), a known teratogen, causes a number of craniofacial defects. Retinoic acid is normally present in the embryo in low amounts but defects can result when excess or deficiency of RA occurs. Retinoic acid acts through its nuclear receptors that in turn bind to different DNA regions directly affecting the expression of some genes, such as homeobox genes, growth factors, oncogenes and ECM molecule genes. In facial morphogenesis, medial nasal mesenchyme exposed to retinoic acid do not develop *in vivo*

(Wedden, 1987; Tamarin et al., 1987), exhibiting an effect resembling that seen after medial nasal process excision. Recent studies involving injection of retinoic acid in pregnant mice at the time of facial development revealed that the RA effect was time dependent. When injected at gestational day 8.25 of mouse embryos, defects of both branchial arches and frontonasal process occurred, while injection at gestational day 10 results in minor branchial arch malformations (Webster et al., 1986; Grant et al., 1997). The same results were recapitulated using rat embryos *in vitro* (Webster et al., 1986). Cell division might be one mechanism of facial growth that is affected, although one should not rule out that teratogens may also affect cell migration (Patterson, Minkoff and Johnston, 1979), ECM synthesis, ECM degradation or a combination of all. It is known that RA suppresses the expression of the collagenase (MMP-1) gene (Pan, Eckhoff and Brinckerhoff, 1995), therefore possibly inhibiting matrix degradation. In chicks, the specific affects of RA on matrix production and cell proliferation of mandibular and medial nasal processes were examined by analysis of $^{35}S$ sulfate, $^{3}H$ thymidine and $^{3}H$ proline incorporation (Sakai and Langille, 1992). Low levels of RA stimulated cartilage matrix production in mandibular but not the medial nasal process as determined by the patterns of sulfate incorporation. At higher levels, sulfated proteoglycans were reduced in both processes. At the same time, RA induced incorporation of proline in the mandibular but not the medial nasal process. The differences observed might reflect the different origins of these two groups of cells within the neural crest cells. It is also known that RA plays a role in homeobox gene expression in the developing head (Studer et al., 1994; Helms et al., 1997). These reports show that high doses of RA can affect the growth of frontonasal and maxillary processes by inhibiting the expression of *shh* and *patched*

homeobox genes disrupting the epithelial-mesenchymal interactions. In addition, methotrexate and aminopterin, have produced similar effects in mouse embryos *in vivo* (Burk and Sadler, 1983; Darab et al., 1987).

Most studies of alterations of ECM in facial development have been confined to known teratogens such as DON and RA. Although they seem effective in producing clefts, their precise mechanisms of action have not been elucidated, complicating the analysis of the results obtained. Despite this major shortcoming, results from these teratogen studies suggest that normal temporo-spatial distribution and production of matrix molecules are required for normal morphogenesis.

## Proteinase Degradation of the Extracellular Matrix

The matrix metalloproteinases are a family of enzymes that can digest at least one ECM component (for a review, Parks and Mecham, 1998). Catalytic activity of these enzymes requires a zinc ion at the active site. There is a second zinc ion in some MMPs and a calcium ion that helps stabilize the tertiary structure of the enzymes (Lovejoy et al., 1994). The combined activation of various matrix metalloproteinases (MMPs), plasminogen activators (PAs) and tissue inhibitors of matrix metalloproteinases (TIMPs) is essential for the efficient turnover of the ECM (Matrisian, 1992; Toumir et al., 1994). They are inhibited by chelating agents, natural and synthetic inhibitors, and are secreted as proenzymes that require proteolytic activation by other proteases *in vivo* or by trypsin and 4-aminophenylmercuric acetate (APMA) *in vitro*. Four-aminophenylmercuric acetate (APMA) works by inducing a conformational change in the zymogen, which allows intramolecular self-cleavage (Nagase et al., 1990).

There are four subclasses of MMPs:

(1) interstitial collagenases (MMP-1 and MMP-8), which degrade collagens I, II and III;

(2) gelatinases (MMP-2 and MMP-9), which degrade collagens IV, V, VII, X, fibronectin (Fn) and gelatins;

(3) stromelysins (MMP-3, MMP-7, MMP-10, MMP-11 and MMP-12), which act on proteoglycans, laminin (Ln), fibronectin, collagens III, IV and V, and gelatins;

(4) membrane-type matrix metalloproteinases (MT1-MMP and MT2-MMP), recently identified, which are bound to the cellular membrane and can also mediate the activation of pro-MMP-2 in the cell surface (Takino et al., 1995).

Most recently, a novel matrix metalloproteinase, MMP-19 has been characterized (Pendás et al., 1997). It is thought to be a unique MMP due to different structural characteristics, but its activity is blocked by TIMP-2 and EDTA, and it is expressed in human placenta, lung, pancreas, ovary, spleen and intestine.

**Gelatinases**

Matrix metalloproteinase-2, also called gelatinase A or 72 kDa type IV collagenase, degrades gelatin, collagens IV, V, fibronectin, collagens VII, and X (basement membrane), in order of preference (Birkedal-Hansen, 1995). It is usually found as a 72 kDa protein (68 kDa intermediate and 66 kDa as proteolytic end product). In rat tracheal tissue 68 kDa and 60 kDa isoforms have been isolated (Lim et al., 1995). In tooth development, the isoform of the MMP-2 is primarily isolated at 72 kDa (Heikinheimo and Salo, 1995). Matrix metalloproteinase-2 is activated at the cell surface and retained

there through interaction with a receptor like molecule (Monsky et al., 1993). It is usually found in complex with TIMP-2, resistant to activation by plasma proteins, but their interaction is a controversial subject. Some authors have demonstrated that TIMP-2 inhibits the activation of pro-MMP-2 while others suggest that it may facilitate it through the formation of a complex with MT1-MMP (MMP-14) on the cell surface or to another TIMP-2 acting as a receptor. It is thought that MMP-2/TIMP-2 complex may still be active but gelatinase activity is only 10% of the free active MMP-2 (Kleiner et al., 1992; Yu et al., 1996). The transmembrane (TM) domain of MMP-14 is essential for MT1-MMP-MMP2 interaction (Cao et al., 1995). The C-terminal domain of MMP-14 binds to the C-terminal domain of the pro-MMP-2 (Werb, 1997). It can also act as a tissue inhibitor of metalloproteinase 2 (TIMP-2) receptor in the cell surface by its N-terminal domain. MT1-MMP makes an excellent substrate for serine proteases, the same sequence is found in collagenases and stromelysins that can be activated by plasmin (Strogin et al., 1995). It has been demonstrated that MT1-MMP is activated in the Golgi by a furin family protease or on the cell surface by proteinases such as plasmin (Sato et al., 1994; Okumura et al., 1997).

Pro-matrix metalloproteinase-2 appears to be constitutively expressed by many cell types (unlike other MMPs) and its message RNA is not easily induced by 12-O-tetradecanoyl-phorbol-13-acetate (TPA) or interleukin-1α (IL-1α), which has been shown to increase the expression of other MMPs. The promoter lacks activating protein-1 (AP-1) or polyomavirus enhancer-binding protein-3 (PEA-3) sites and also transforming growth factor-β (TGF-β) inhibitory element (Yu et al., 1996).

Matrix metalloproteinase-9, also called gelatinase B or 92 kDa type IV collagenase, degrades collagens IV, V and XI, laminin, elastin, entactin, aggrecan core protein and cartilage link protein (Birkedal-Hansen et al., 1993). Matrix metalloproteinase-9 is 92 kDa in humans or 105 kDa in rodents (Tanaka et al., 1993). Matrix metalloproteinase-9 forms a complex with tissue inhibitor of metalloproteinase-1 (TIMP-1). When the pro-MMP-9/TIMP-1 complex is treated with APMA or trypsin, no gelatinolytic activity is detected, although pro-MMP-9 is converted to lower molecular weight forms, which correspond to activated forms (Ogata, Itoh and Nagase, 1995). A complex with pro-MMP-1 can be formed which does not allow MMP-9 to bind to TIMP-1. The complex MMP-1/MMP-9 is readily activated by MMP-3. Matrix metalloproteinase-2 also seems to activate pro-MMP-9, resulting in 86 kDa (intermediate) and 67 kDa (active) isoforms (Fridman et al., 1995). At the same time, activated MMP-7 can also activate pro-MMP-9 in human rectal carcinoma cells (Imai et al., 1995). In human neutrophils, MMP-9 is detected at 57.5 kDa (unglycosylated proenzyme), 49 kDa and 41.5 kDa as active forms (Bu and Pourmotabbed, 1995).

**Stromelysins**

The stromelysins are MMP-3, MMP-7, MMP-10, MMP-11 and MMP-12. They degrade fibronectin, elastin, laminin and proteoglycans and have limited activity on nonhelical regions of collagens IV, V, VIII, IX and the amino terminal of collagen I (Birkedal-Hansen, 1995). Matrix metalloproteinase-3 can activate MMP-9 to a greater extent than APMA (Ogata, Enghild and Nagase, 1992). Matrix metalloproteinase-3 (human stromelysin 1) is a pro-enzyme of 57 kDa and 60 kDa (unglycosylated and

glycosylated) and has a molecular weight of 45 kDa as the active form. It has been detected in some mesenchymal tissues (Okada et al., 1992).

Matrix metalloproteinase-7, also called matrilysin or PUMP-1, is distinct in that it contains only the catalytic domain required for activity, in contrast to the other members of the family, which contain additional carboxyl-terminal domains (Gaire et al., 1994). It is 28 kDa in the latent form, 21 kDa and 19 kDa as active forms. It degrades a wide range of substrates such as fibronectin, laminin, proteoglycans, elastin, gelatin, collagens IV and IX, aggrecan, entactin and small tenascin (Birkedal-Hansen, 1995). Human and mouse MMP-7 share 75% of protein sequence similarity (Wilson et al., 1995). MMP-7 can be activated by APMA, trypsin and also by MMP-3. In turn, MMP-7 can activate pro-MMP-1 and pro-MMP-9 (Fridman et al., 1995). The expression of MMP-7 is almost exclusively found in epithelial cells (Wilson et al., 1995; Saarialho-Kere, Crouch and Parks, 1995).

**Collagenases**

The collagenases are MMP-1, MMP-8 and MMP-12. They cleave native triple helical collagen in each $\alpha$ chain, generating two fragments. These fragments denature at 37°C to form gelatin, and although the collagenases can degrade gelatins to a certain extent, the gelatinases are the ones to do so more often and faster. Matrix metalloproteinase-1, also known as interstitial collagenase or fibroblast collagenase, degrades collagens I, II, III, VII and X (Welgus et al., 1990), proteoglycan link protein and gelatins. Many studies have shown that MMP-1 is not expressed until gestational day 14.5 or 15 in mouse embryos (Gack et al., 1995; Mattot et al., 1995). Its expression can be correlated with the onset of bone or cartilage formation.

The Matrix metalloproteinases can be inhibited by their natural inhibitors, TIMPs, by chelating agents, such as EDTA, and by specifically synthesized inhibitors such as GM6001 (galardin) (Boghaert et al., 1994; Odake et al., 1994; Shams, Hanninen and Kenyon, 1994), a hydroxamate or by Batimastat (Sledge et al., 1995), a new class of agents specifically designed to inhibit MMP activity. There are four members of the class of TIMPs: TIMP-1, TIMP-2, TIMP-3 (Leco et al., 1994) and the recently isolated TIMP-4 (Leco et al., 1997). All TIMPs are glycoproteins, each with a different molecular weight: TIMP-1 is 28 kDa (glycosylated); TIMP-2, 20 kDa (unglycosylated); TIMP-3, 24 kDa; and TIMP-4, presumably 23 kDa. They form a non-covelantly linked 1:1 complex with the activated form of MMPs and inhibit their proteolytic activity through a mechanism that is not entirely clear. It has been shown that TIMP-1 occupies the entire length of the active site of MMP-3 therefore blocking its activity (Gomis-Ruth et al., 1997). Several lines of evidence suggest TIMPs play a role in growth and development (Brenner et al., 1989). Messanger RNA transcripts for collagenase, stromelysin and TIMPs were detected in mouse embryos suggesting their function during growth, development and implantation of mammalian embryos (Behrendtson, Alexander and Werb, 1992).

Regulation of the MMPs is complex and can occur at different sites: intracellularly, at the transcriptional or translational level, extracellularly, by activation of the proenzyme and binding of the inhibitors. Since it is likely that little or no active matrix proteinase is free in the tissue in physiologic remodeling, MMPs are either released in a latent form to be activated by another protease, or have been blocked by inhibitors.

## Matrix Metalloproteinases in Other Embryonic Systems

A large and growing body of evidence suggests that MMPs play an important role in the remodeling of ECM in various embryonic developing systems. The presence of MMPs, TIMPs and their mRNAs was described in early blastocyst stage of mouse embryos using zymograms and RT-PCR (Brenner et al., 1987). The addition of TIMPs to the culture medium of early mouse embryos slowed development but did not inhibit it completely. Matrix metalloproteinase-9 has also been detected in murine and human osteoclasts where it is required for removal of collagen during resorption remodeling of bone (Reponen et al., 1994). Matrix metalloproteinase-9 mRNA has also been found during mouse neurogenesis (Canete-Soler et al., 1995). At day 11, MMP-9 message was localized in the epithelium of nasal pit and distributed within the mandibular process among other regions of the mouse head. Elevated expression of MMP-9 in cytotrophoblasts of developing embryos has been detected as well (Librach et al., 1991), suggesting that this enzyme may play an important role in cellular migration, invasion and tissue remodeling.

Matrix metalloproteinase-2 has also been found in numerous developing systems. The occurrence and distribution of MMP-2 in human embryonic tissues also suggests that ECM remodeling and degradation by proteinases is a physiological event in association with ECM deposition during development (Casasco et al., 1995). Tracheal tissue morphogenesis involves the penetration of epithelial cells into the submucosa, a process that requires digestion of the basal lamina and the surrounding ECM. Bovine tracheal

tissue cells produce and secrete MMP-2 (Tournier et al., 1994). Its localization in tracheal tissue and gland acini was demonstrated using antibodies. Matrix metalloproteinase-2 was upregulated in epithelial and stromal cells suggesting that it probably plays a role during rat tracheal gland morphogenesis (Lim et al., 1995). The message for MMP-2 is expressed during murine embryogenesis. Using in situ hybridization, intense expression was found in day 10-15 embryos in mesenchymal tissue of kidney, lung and epithelium of the submandibular gland (Reponen et al., 1992). The overall results of MMP-2 localization studies showed that MMP-2 is primarily expressed in mesenchymal cells with the exception of gland morphogenesis (salivary, tracheal).

There is limited knowledge about the physiological role of MMP-7. However, there is evidence for it having a role in fetal development. The synthesis of MMP-7 in developing human mononuclear phagocytes was reported (Busiek et al., 1992). Matrix metalloproteinase-7 was also found in germinal basal cells during human fetal skin development (Karelina et al., 1994). In the mouse, MMP-7 appears to be primarily expressed by epithelial cells or epithelial derived cells and in tumors, by parenchymal cells rather than stromal cells (Wilson et al., 1995; Wilson and Matrisian, 1996, 1998). Due to its localization, MMP-7 has been associated with basement membrane degradation in neoplastic lesions (Wilson et al., 1995) and in many embryonic systems (Busiek et al., 1992; Karelina et al., 1994; Saarialho-Kere, Crouch and Parks, 1995).

Formation and degradation of dental basement membrane are important for tooth development. Expression of MMP-2 and MMP-9 was observed in human fetal tooth development (Heikinheimo and Salo, 1995). During later stages, high levels of MMP-2 mRNA were confined to differentiating and secretory odontoblasts. This indicates that

MMP-2 might participate in both remodeling of the enamel organ-dental papilla basement membrane and its final degradation.

**Other Morphogenetic Factors**

Besides MMPs, another family of proteinases, the plasminogen activator (PA) family, may influence ECM degradation through activation of the MMPs or by directly acting on the matrix. Two different enzymes have been identified in mammals as members of the PA family: urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA). Like PAs, plasmin has also been implicated to have a role in ECM degradation (Vassalli, Sappino and Belin, 1991). Plasminogen is the preferred substrate for PAs whereas fibrin is the preferred substrate for plasmin but most matrix proteins can also be cleaved by these enzymes. Plasminogen activators convert plasminogen into plasmin and, in addition, plasmin is one of the activators of metalloproteinase precursors (He et al., 1989). Plasminogen activator catalyzed activation of plasminogen results in a dramatic amplification of the proteolysis of the matrix components. The expression and activity of uPA has been demonstrated in preimplantation rat embryos (Zhang et al., 1994), suggesting that embryonic uPA may be involved in early embryo development and implantation.

Recent studies involving genetic knockouts of uPA, tPA and plaminogen activator inhibitor-1 (PAI-1) (Carmeliet et al., 1995) produced mild phenotypes, suggesting that these proteases have a limited role in regulatory development. It might be possible that their functions have been substituted by other similar proteins since there seems to be a high level of gene redundancy that still remains to be elucidated.

## The Proposed Role of MMPs in Facial Formation

In normal primary palate and face morphogenesis, closure is dependent on the lateral nasal processes making contact with the medial nasal process. Besides the rapid growth and high rates of cell proliferation, this contact is enhanced by the morphogenetic movements of the lateral process, initiated as a curling forward of the lateral portion to form the nasal wing. These morphogenetic movements result from specific changes in tissue architecture involving temporo-spatially localized alterations in the distribution of the ECM molecules. Proteinase mediated degradation of the ECM molecules likely plays a role in these changes. This suggests that matrix proteinases that degrade extracellular matrix are necessary for these events to occur. The role of MMPs, their activators and inhibitors in modulating the ECM environment during this morphogenetic event has not been explored.

The MMPs and PAs have also been shown to play a role in the metabolism of the ECM in development. We suggest that they are probably involved in facial morphogenesis. Several MMPs, TIMPs and their messages are present in the facial processes, including MMP-2, MMT1-MMP and MMP-9 (Kinoh et al., 1996; Iamaroon et al., 1996, Chin and Werb, 1997), and seem to be temporally regulated over the course of murine facial development. At this time, the processes are a collection of undifferentiated cells that will only begin to differentiate at late day 11, forming specialized organs such as teeth and later on, specialized tissues such as cartilage and bone. Thus, the MMPs detected should be produced only by the mesenchymal and epithelial cells of the facial processes.

It has been demonstrated that many tumor types express MMPs. It is important to note that expression within a tumor type does not recapitulate its fetal expression. For example squamous cell carcinomas of the skin (Pyke et al., 1993) and lung (Canete-Soler, Litzky and Muschel, 1994) express MMP-9 but only the lung demonstrates expression of MMP-9 during embryogenesis. Therefore, the fetal expression pattern does not predict which tumors in adults will express MMPs and vice-versa.

It is interesting to discuss why two different types of matrix metalloproteinases (MMP-2 and MMP-9) with similar substrate specificities are expressed at the same time during morphogenesis. Results of transgenic mouse experiments with knock-outs for MMP-3, MMP-7, MMP-9 and MMP-12 and overexpression of MMP-1 and MMP-3 were disappointing (Shapiro, 1997) since no dramatic phenotypes were produced. These results seem to give an idea the redundancy of MMP function. One or more enzymes can take over and compensate after one MMP is knocked out. The characterization of their genes have demonstrated that each enzyme may have a different control of expression (Huhtala, Chow and Tryggvason, 1990; Huhtala et al., 1991). By responding to different elements (for example, growth factors or the presence of inhibitors), the cell can regulate the expression of the different enzymes and change its surrounding environment.

The interactions of the ECM molecules, MMPs, PAs and their inhibitors must be intrinsically regulated in normal morphogenesis. These factors may be controlled by differential spatial and temporal localization within a given tissue. The initial step in elucidating the role of these molecules in mid-facial and primary palate morphogenesis is to determine the temporal appearance and distribution of matrix metalloproteinases, their target ECM molecules, and their messages. These data will permit the identification of

specific times in facial morphogenesis for optimal targeting of perturbation experiments that will further define the role of matrix metalloproteinases in the process.

## Cleft Lip and Palate

Although there is considerable knowledge of the development of various craniofacial malformations, less progress has been made in research related to the mechanisms involved in development and fusion of the upper facial processes. Alterations of the developmental sequence of these processes lead to the most common major craniofacial malformations, cleft lip and/or palate. It is estimated that one of every 700 live births in the United States has a cleft lip or palate or both. Since embryological studies demonstrated that the lip and the palate close at different times, it is hypothesized that cleft lip and cleft palate may arise through different mechanisms or processes.

Clefts have different etiologies: genetic, with transforming growth factor-α (TGFα) being the candidate gene in certain families with predisposition to cleft palate (Ardinger et al., 1989), and environmental, which is thought to account for the majority of the occurrences. Knock-out experiments of transforming growth factor β3 (TGFβ3) showed that null homozygous mutant animals suffer from cleft palate (Kaartinen et al., 1995). Recent studies have suggested that a combination of genetic background of the affected person and of the mother and environmental agents have to be present for the occurrence of nonsyndromic oral clefts (Maestri et al., 1997). These are complex birth defects characterized by an uncertain mode of inheritance, incomplete penetrance, and heterogeneity within and among populations. Environmental factors can be divided into

five groups: (1) infectious agents, (2) irradiation, (3) drugs, (4) hormones, and (5) nutritional deficiencies (Ten Cate, 1989).

The use of inbred mice A/J, A/WySn and C57BL6/J strains (susceptible to clefts after use of teratogens) is an excellent tool for the identification of genes or chromosomal locations involved in clefting. A number of candidate genes have been found by performing a genome wide search for loci in mouse (Diehl and Erickson, 1997). Among these are:

- eight collagen genes and several extracellular matrix components;
- twenty oncogenes or tumor suppressor genes;
- sixteen genes related to detoxification of teratogens such as glutathione S-transferase;
- homeobox genes such as Msx1, Msx2 and Pax9; five genes related to retinoic acid, including β and γ (α, involved in spontaneous cleft, lies outside);
- growth factors and receptors, including epidermal growth factor (EGF), epidermal growth factor receptor (EGFR), fibroblast growth factor receptor (FGFR) and TGFβ3.

The authors suggested that multiple loci contribute to facial clefts and some of these loci can cause susceptibility to both cleft lip and cleft palate but with different parental strain alleles contributing to two different forms.

## Significance of This Study

Facial morphogenesis is dependent on ECM synthesis and accumulation. It is tightly regulated by the interaction of the proteins that degrade the ECM in a given time and space. Current literature suggests that matrix-degrading enzymes play a role in many

developmental processes. We suggest that facial development is one of these processes. Molecules known or expected to be involved in various aspects of normal development in animal models have been tested in humans with defects and have been shown to be involved in a number of common malformations. This study allows determination of whether matrix proteinases play a role in primary palate and mid-face morphogenesis. Our study may provide insights into normal and abnormal facial development particularly relevant to the occurrence of cleft lip. By identifying the molecules that may play a role and providing indications on how they may alter development, we can have a better understanding of the whole process. This may lead to strategies for preventing the action of perturbing agents and compensating for defective genes.

# MATERIALS AND METHODS

## Materials

CD-1 mice were purchased from Charles River Laboratory. Microfast kits for RNA extraction were purchased from Invitrogen (Carlsbad, CA). Reverse transcriptase-polymerase chain reaction (RT-PCR) Gene-Amp RNA kits were obtained from Perkin-Elmer-Cetus (Emeryville, CA). Antibodies to laminin (A/B) were purchased from Chemicon (Temecula, CA), as were positive controls for gelatinases A/B. Monoclonal antibodies to fibronectin were purchased from NeoMarkers (Fremont, CA). Another set of antibodies to laminin (B1/B2) was obtained from UBI (Lake Placid, NY). Antibodies to MMP-2, MMP-3 and MMP-9 were gracious gifts from Dr. David Muir (University of Florida)(Muir, 1994; 1995). Secondary Alkaline Phosphatase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG were purchased from American Qualex (San Clemente, CA). Anti-sheep IgG (whole molecule) alkaline phosphatase-conjugated second antibody was purchased from Sigma (St. Louis, MO). Culture media and supplements were obtained from Gibco BRL/Life Technologies (Gaithersburg, MD). Culture dishes and Transwell-Clear supports were acquired from Costar (Cambridge, MA). Galardin® (GM6001), a dipeptide analogue with the structure of N-[2(R)-2-(hydroxamido carbonylmethyl)-4-methylpentanoyl]-L-tryptophanemethylamide synthesized as described (Grobelny D, Poncz L, Galardy R, 1992 and unpublished data) was a gift from

Dr. Greg Schultz (University of Florida). Tissue inhibitor of metalloproteinase-2 (TIMP-2) for culture experiments was acquired from Boehringer Mannheim (Indianapolis, IN).

**Animals and Tissue Preparation**

The presence of a vaginal plug following overnight mating was taken to indicate pregnancy and designated gestational day 0 (gd 0). At days 9, 10, 10.5 and/or 11, the pregnant mice were killed by cervical dislocation. Embryos and facial processes were dissected under the light microscope using microinstruments. Besides date of pregnancy, total appearance, crown-rump length, facial appearance and limb development were used as physical features in the developmental classification of the embryos in each litter through their comparison to anatomical charts (Kaufman, 1992; Theiler, 1989). Maxillary and mandibular processes were obtained from gestational days 10 and 11. Lateral and medial nasal processes were obtained from day 11. Total RNA was extracted by using Micro-Fast Track kit. Tissues were homogenized for immunoblots and zymography in non denaturing buffer (200 mm NaCl, 50 mM Tris, 5 mM $CaCl_2$, pH 7.6 and 0.1% Triton X-100) (Carol Brenner, personal communication) and DNA was enzymatically digested. Samples were maintained in -80°C freezer. Protein concentration was determined by Bio Rad Protein Assay.

## Zymography

Facial processes samples containing 2 to 10 μg of total protein were mixed with Laemmli sample buffer and were separated under non-reducing conditions on 10% polyacrylamide gels containing gelatin, α-casein or α-casein with 0.04 U/ml plasminogen as substrates (Adler, Brenner and Werb, 1990; Chin, Murphy and Werb, 1985). The gels were incubated at 37°C overnight in buffer (50 mM Tris, 5 mM $CaCl_2$, 150 mM NaCl, 0.02% $NaN_3$, pH7.8) with or without the inhibitors; 4mM ethylenediaminetetraacetic acid (EDTA), 10 mM phenanthroline, 20mM phenylmethylsulfonyl fluoride (PMSF), aprotinin (1μg/μl). Following removal of SDS by washing in buffer containing 0.1% Triton-X 100, the gels were stained with Coomassie blue. After succeeding destaining, enzyme activity appears as a clear band against a blue background of undigested protein.

Gels were photographed and then dried. Dry gels and their pictures were scanned and analyzed using Gel Pro Analyzer program. This program gives the approximate molecular weight for each band corresponding to regions of gelatinolytic activity and compares to the known molecular weight standards run on the same gel. It also performs a densitometric analysis that gives the ratio of intensity of each active band in the gel compared to the others. Band sizes and activity intensities were estimated using this program.

One mM of 4-aminophenylmercuric acetate (APMA) was used for activation of gelatinases, by incubating the samples overnight at 37°C. Four-aminophenylmercuric acetate (APMA) activates matrix metalloproteinases by cleaving the propeptide and subsequently exposing the active site.

## Reverse Zymography

Reverse zymography is a useful technique that allows visualization of the presence of natural inhibitors. To detect TIMP inhibitory activity, gels were performed using collected conditioned media at 23.5% (vol) in 10% SDS-PAGE gelatin gels. The gels were incubated at 37°C overnight in buffer (50 mM Tris, 5 mM $CaCl_2$, 150 mM NaCl, 0.02% $NaN_3$, pH7.8). Following removal of SDS by washing in buffer containing 0.1% Triton-X 100, the gels were subsequently stained with Coomassie blue. Since the whole gel carries media that contains proteinases, a blue band of non-degradation appears in the place where the inhibitor is located, demonstrating the inhibitor's presence and molecular weight.

## Immunoblotting

Forty μg of each facial process sample was dissolved in a sample buffer containing 5% β-mercaptoethanol, separated on 10% SDS-PAGE gels and then transferred to a nylon membrane (Millipore, Bedford, Mass). The membrane was incubated with blocking solution (5% dry milk/ 3% bovine serum albumin (BSA)) in TTBS buffer (10 mM Tris-HCl pH 7.4, 200 mM NaCl, 0.02% $NaN_3$, Tween-20) for 3 hours at room temperature prior to overnight incubation with primary antibody diluted in the blocking solution. The primary antibodies used were: rabbit IgG anti-human MMP-2 (made against peptide 475-490 residues, gift from Dr. David Muir), sheep anti-human MMP-9 (gift from Dr. David Muir), sheep anti-human MMP-3 (gift from Dr. David Muir) (Muir, 1994; 1995), mouse monoclonal anti-fibronectin (NeoMarkers, Fremont,

CA), rabbit polyclonal anti-mouse laminin B1/B2 (Upstate Biotechnology Incorporated), rabbit polyclonal anti-mouse laminin A/B and rabbit anti-mouse collagen IV (Chemicon, Temecula, CA). Antibodies against MMP-2 were raised against the sequence MGPLLVATFWPELPEK corresponding to amino acid residues 475-490 near the carboxy terminus of MMP-2 (Muir, 1994). This antibody binds to both the proform and the activated form of the MMP-2 enzyme but demonstrates a variable detection of the proform in immunoblots (Collier et al., 1988; Muir, 1994). The antibody against MMP-3 was raised against the sequence DPNAGKVTHILKSN, corresponding to the residues 457-470 of the rat MMP-3 at the carboxy terminal (Muir, 1994). Blots were washed three times with TTBS for 45 min. The membranes were incubated with the respective secondary antibodies overnight, followed by washing step described above. Bands were detected by a visualization protocol coupling alkaline phosphatase to BCIP and NBT as substrates, a fast procedure that allows visualization within 30 min (average) with sensitivity as low as 100 pg of protein. In addition to tissue samples from the different facial processes, controls for antibody specificity were comprised of no incubation with the primary antibody. Negative control samples used tissues homogenates that did not contain the target protein while positive controls included tissue preparations that contained known amounts of target antigen, such as for MMP-2 and MMP-9 positive controls from Chemicon (Temecula, CA) or isolated human placenta (Shimamori, Watanabe, Fujimoto, 1995).

**Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)**

Reverse Transcriptase-Polymerase Chain Reaction reactions were performed to detect the presence of specific messages. The nucleotide sequences for mouse genes were retrieved from Genbank and primers were specifically designed using MacVector software program version 6.0.1 (Oxford Molecular Ltd., San Jose, CA) and synthesized in the University of Florida DNA Synthesis Core Facility. We designed specific primers for MMP genes, MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, TIMP-1, TIMP-2, tPA and uPA (table 1). CopyDNA was synthesized in 20 μl reactions containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl buffer; 5mM $MgCl_2$; 1 mM of each dNTP; 1 U of Rnase inhibitor; 2.5 U of Reverse Transcriptase; 2.5 μM of random hexamers primers, and ≤ 1 μg of total RNA. The reaction was carried out at 42°C for 15 min, 99°C for 5 min and 5°C for 5 min. DNA amplification was performed in 100 μl reactions containing 20 μl of initial RT reaction in 10 mM Tris-HCl (pH 8.3), 50 mM KCl buffer; 2 mM of $MgCl_2$; 2.5 U of Taq polymerase and 30 mM of each 5'and 3'primers. The PCR reaction was carried out at 94°C for 4 min for the initial hot start, 94°C for 1 min, 60°C for 1 min and 72°C for 1 min for 35 to 40 times and 72°C for the final 7 min. The annealing temperature of 60°C was adjusted according to the conditions set for each reaction and each targeted message.

Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) or β-actin were used as positive controls for reactions. Each product was further analyzed by southern blot technique to confirm its identity using an internal probe. The primers used are shown below.

**Table 1:** MMP, PA and TIMP Primers.

| Gene | 5' | 3' | bp |
|---|---|---|---|
| MMP-1 | GCCATTACCAGTCACCGAGGA | GGAATTTGTTGGCATCAC TCTCAC | 467 |
| MMP-2 | GGCCATGCCATGGGGCTGGA | CCAGTCGGATTTGATGCT TC | 762 |
| MMP-3 | CTGGAGGTTTGATGAGAAGA | AAACCAGCTGTTGCTCTT CA | 207 |
| MMP-7 | GTGAGGACGCAGGAGTGAAC | ACAGGTGCAGCTCAGGAA GG | 309 |
| MMP-9 | AGGCCTCTACAGTCTTTG | CGTCCTTTCTTGTTGGAC TG | 825 |
| uPA | GTGGAGAACCAGCCCTGGT | GGCAGGCAGATGGTCTGT TAT | 348 |
| tPA | TCCACCTGCGGCCTGAGGCAA T | CACACTCTGTCCAGTCAG GGAG | 445 |
| TIMP-1 | TGCCTCATCCCATCCCCACAA | AGGGGTGTCAGAGGGTGA CAA | 530 |
| TIMP-2 | GAGCCAAAGCAGTGAGCGAG A | GGTACCACGGCGAAGAAC CAT | 400 |
| β-ACTIN | TCTACAATGAGCTGCGTGTG | GAAGTACTCCATCAGGCA GGTC | 304 |
| G3PDH | TGAAGGTCGGTGTGAACGGAT TTGGC | CATGTAGGCCATGAGGTC CACCAC | 983 |

## Southern Blot Analysis

To confirm the identity of the amplicons generated by PCR, the products were separated in a 1.5% agarose gel and transferred to a nylon membrane (Boehringer Mannheim, Indianapolis, IN). Internal probes were specifically designed from gene sequences retrieved from Genbank and digoxigen labeled as per manufacturers instructions (Boehringer Mannheim, Indianapolis, IN) (Table 2). After hybridization, alkaline-phosphatase anti-digoxigen antibody was applied to the membrane, followed by a chromogenic substrate. The probes used are shown in Table 2.

**Table 2:** MMP, PA and TIMP Probes.

| Gene | Sequence |
|---|---|
| MMP-1 | TGAGGCTGAGCTCTTTTTGACAAAGTCCTT |
| MMP-2 | GACAAGCCCACAGGTCCCTTGCTGGTGGCC |
| MMP-3 | TAGCTGAGGACTTTCCAGGTGTTGACTCAA |
| MMP-7 | GAGACTACTCAGAAGACTTCAGTCTTACAA |
| MMP-9 | GATCCAGGGCGCTCTGCATTTCTTCAAGGA |
| uPA | TCCTCCCTCCTTTAAATGTGGTGGGAGTCT |
| tPA | GGAAAGGAGGAGCAGACATTCGAGATCGAA |
| TIMP-1 | GGGCAGGGCAGGATGGAGTAGGGGATGGTT |
| TIMP-2 | CCCATCAAGAGGATTCAGTATGAGATCAAG |

**Competition-Based Quantitative RT-PCR**

Competition-based Q-RT-PCR allows correct calculation of the number of mRNA molecules in a sample. This new method is based on the fact that up and downstream oligonucleotide primers will compete equally for authentic cellular cDNA molecules and synthetic template cDNA from the reverse transcription reaction granted that the synthetic template contains the identical complimentary sequences found in the original cellular mRNA. Therefore, a competition will be established. The log of the ratio of band intensities within each lane is plotted against the log of the copy number of template added per reaction. When the ratio of template and target intensities is equal to one, the quantity of target messages is determined. A correct standard curve can be considered a strong argument for equal rates of amplification (Raeymaekers, 1994; 1995).

The ECM supertemplate (Tarnuzzer et al., 1996) was diluted in known concentrations of $2.16 \times 10^6 - 2.16 \times 10^{10}$ copies and used in reverse transcriptase

reactions to compete with the gene of interest. For the initial RT step, 1µl of the diluted supertemplate and 1µg of total RNA were added to each reaction and cDNA was synthesized in a series of standard 20µl reactions each containing 1.5 mM $MgCl_2$, 200 µM dNTPs, 200 U of Reverse Transcriptase, 50 U/ml of RNase inhibitor, 2.5 µM of oligo d(t) primer, 1 µl of the diluted supertemplate and 1 µg of total RNA. The reaction was carried out at 42°C for 15 min, 99°C for 5 min and 5°C for 5 min. Polymerase chain reaction was performed for each ECM gene for DNA amplification in 100 µl of total reaction containing 10 µl the initial RT reaction, 50 pmol of each primer 5'and 3', 1.5 mM $MgCl_2$, 200 µM dNTPs and 2.5 U of Taq polymerase. This reaction was carried out at 94°C for 4 min for initial hot start, 94°C for 1 min, 58°C for 1 min and 72°C for 1 min for 35 to 50 cycles and 72°C for the final 7 min of extension in a Biometra thermocycler.

The following table shows the expected product sizes for each gene.

**Table 3:** ECM Supertemplate, expected product sizes.

| | ECM Template (bp) | Cellular mRNA (bp) |
|---|---|---|
| **Fibronectin** | **347** | **768** |
| **Laminin B2** | **344** | **677** |
| **Collagen III** | **338** | **522** |
| **Collagen I** | **335** | **769** |
| **Collagen IV** | **335** | **743** |
| **Laminin B1** | **335** | **594** |
| **Elastin** | **338** | **704** |

## Analysis of RNA Results

Gels containing the PCR products were photographed and scanned. The intensity of staining was determined using NIH Image program version 1.54. These intensity values were normalized for their molecular weight. The log of the ratio of the band within each lane was plotted against the log of the copy number of template added per reaction. Quantity of target messages was determined where the ratio of template and target band intensities equals one (Tarnuzzer et al., 1996).

## Statistical Analysis of Q-RT-PCR Data

All data were statistically analysed by both Kruskall-Wallis one-sided ANOVA. The null hypothesis stated that all means were equal while the alternative hypothesis stated that at least two of the means were different. Data were considered statistically significant at $p < 0.05$.

## Embryonic Heads Culture

Embryonic heads from gestational days 9, 10 and 10.5 were cultured in 24-well dishes using Transwell-Clear permeable supports (6.5mm, 3.0 μm) with BGJb medium supplemented with 0.1 mg/ml ascorbic acid, 10 mM Hepes, penicillin-streptomycin, pH 7.4 (Slavkin et al., 1989; Shum et al., 1993; Chin and Werb, 1997) and 20% fetal bovine serum. Cultures were maintained from 9 to 12 days at 37°C and 5% carbon dioxide with

media changed every 48 hs. Working dilutions of all inhibitors and comparable controls (dimethyl sulfoxide (DMSO), methylcellulose in phosphate buffer (PBS), $H_2O$, TIMP-2 buffer and medium alone) were made in culture medium. The following inhibitors were added to the culture: 1,10 phenanthroline (150 µl of solutions of 400mM); galardin (15 µg/ml, 150 µg/ml and 1mg/ml) (Boghaert et al, 1994; Odake et al, 1994); EDTA (1mM and 400 mM); TIMP-2 (0.02 µg/ml, 0.05 µg/ml, 0.1 µg/ml, 0.2 µg/ml, 0.5 µg/ml and 1 µg/ml) (Albini et al, 1991); and aprotinin (1.5 µg/ml and 3.0 µg/ml).

All experiments were performed at least three times on separate occasions with five embryonic heads of gestational ages 10 or 10.5 for each treatment. The progress towards fusion of facial processes was documented using a Nikon camera attached to an inverted microscope.

**Sectioning of Tissues**

Tissues from culture experiments were fixed in vials with 10% buffered formalin for up to four hours at 4°C. Using an automated preparation device, tissues were dehydrated in graded series of alcohol, cleared in xylene, infiltrated and embedded in paraffin. The paraffin molds were left overnight in the -20°C freezer to harden. Paraffin blocks were serial-sectioned at 5-8 µm of thickness. Sections were transferred to subbed slides and placed on a slide warmer at 45°C to stretch the sections for at least 1 hour. Slides were stained with haematoxylin/eosin or stored for subsequent use in immunohistochemistry.

# RESULTS

## Extracellular Matrix Molecules Are Being Synthesized and Degraded at the Time of Facial Formation *In Vivo*

Immunoblots were performed to identify the extracellular matrix composition of the facial processes (Fig. 2). These demonstrated the presence of basement membrane molecules such as collagen IV, fibronectin and laminin in all samples for gestational days 10 and 11. For collagen IV, densitometric analysis of western blots showed an increase of 2.5 fold in protein expression for mandibular process from day 10 to day 11. In contrast, a decrease of 1.2 to 1.5 fold of collagen IV in maxillary, lateral and medial nasal processes from day 10 to day 11 was observed. However, immunoblots for fibronectin and laminin demonstrated a slight increase in the expression of these proteins from day 10 to 11.

To further correlate the presence of specific protein with message RNA synthesis, competition-based quantitative RT-PCR (Tarnuzzer et al., 1996) was performed. Using the calculations described in the Materials and Methods section, the number of steady-state copies per cell of each ECM gene was calculated. A high number of ECM mRNA copies per cell were detected for maxillary, lateral and medial nasal processes of gestational day 11. Collagen I message was absent in all samples for both days. The null hypothesis which states the means are equal on all days analyzed can be rejected, as $p<$ 0.05 for collagens III and IV, for laminin B1 and for fibronectin. For collagen III, there

was a steady state increase of 2.8 fold in message copies/cell for maxillary process from day 10 to 11. For laminin B1, the increase was even higher, 5.7 fold. However, only a slight increase in production of mRNA of ECM components from samples of day 10 to day 11 (maxillary and mandibular processes). For collagen III, there was a steady production of message for fibronectin and collagen IV was observed from gestational day 10 to day 11. The number of copies of mRNA per cell detected in this study was similar to other studies for different tissues (Shim et al., 1997; Macauley et al., 1997). These results are summarized in Figs 3, 4, 5, 6 and 7.

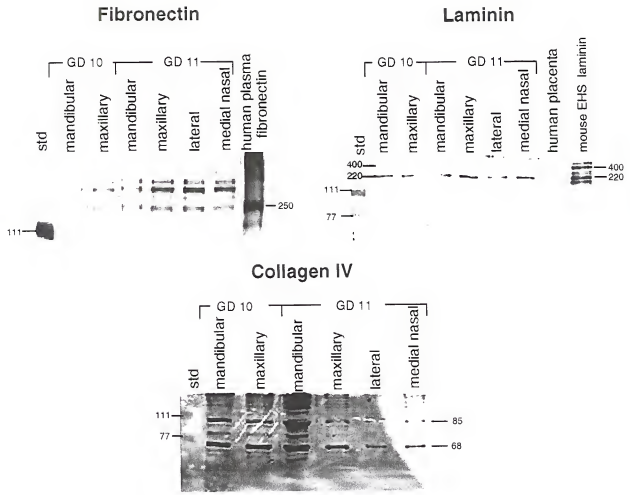


Fig 2. Identification of ECM composition of the facial processes, demonstrating the presence of basement membrane molecules such as collagen IV, fibronectin and laminin in all samples for gestational days 10 and 11. For fibronectin, a band at 250 kDa was detected and co-migrated with human purified plasma fibronectin. Laminin blots demonstrated presence of bands at 220 and 400 kDa, presumably A and B chains of laminin protein. Mouse EHS tumor cells purified laminin was used as positive control. For collagen IV, immunoblots revealed bands at 68 and 85 kDa. Densitometric analysis of western blots showed an increase in collagen IV protein expression for mandibular process from day 10 to day 11 and a decrease in maxillary, lateral and medial nasal processes from day 10 to day 11. Immunoblots for fibronectin and laminin demonstrated a slight increase in the expression of these proteins from day 10 to 11. The molecular weight (kDa) is indicated in the side of the panel. Molecular weight standards are: 111 kDa, Phosphorylase B; 77 kDa, Bovine serum albumin; 48.2 kDa, Ovalbumin; 33.8 kDa, Carbonic anhydrase.

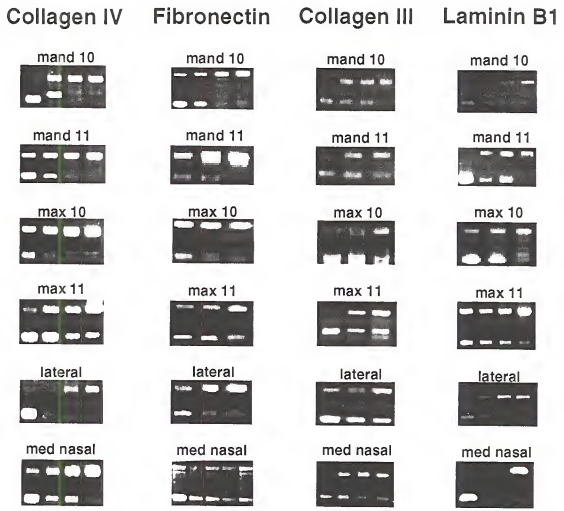


Fig.3. Representative composite data for quantitative RT-PCR. The products size are the same as listed in table 3. The lanes of each agarose gel contain RT-PCR products generated with decreasing numbers of pMATRIX template moving left to right where the upper bands represent the gene and the lower bands represent the template. All reactions were made on three separate preparations of total RNA.

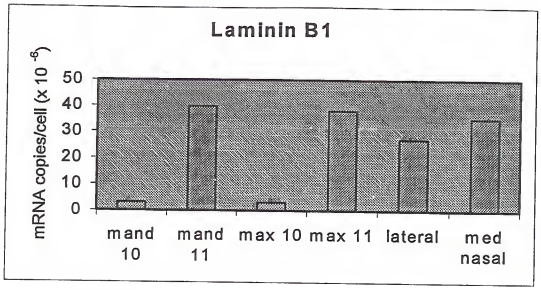


Fig.4. Steady state levels of mRNA copies per cell of facial processes for ECM genes measured by Q-RT-PCR. For laminin B1, there was an increase of 5.7 fold in message copies/cell for maxillary process from day 10 to 11. Similar numbers were obtained using the same technique to investigate pre-implantation rat uterus (Shim et al., 1997). All reactions were made on three separate preparations of total RNA. Values are expressed as means of three determinations ± Standard Error Mean (SEM). SEM values for mand 10, $\pm 5 \times 10^6$; for mand 11, $\pm 2 \times 10^6$; for max 10, $\pm 1 \times 10^6$; for max 11, $\pm 4 \times 10^6$; for lateral, $\pm 2 \times 10^6$; for med nasal, $\pm 3 \times 10^6$.

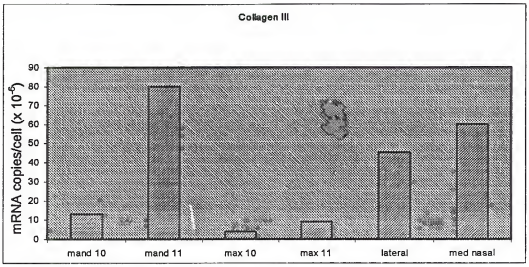


Fig.5. Steady state levels of mRNA copies per cell of facial processes for ECM genes measured by Q-RT-PCR for Collagen III. An increase in the steady state levels of 2.8 fold in message copies/cell was observed for maxillary process from day 10 to 11. All reactions were made on three separate preparations of total RNA. Values are expressed as means of three determinations ± SEM. SEM values for mand 10, $\pm 3 \times 10^6$; for mand 11, $\pm 8 \times 10^6$; for max 10, $\pm 1 \times 10^6$; for max 11, $\pm 5 \times 10^5$; for lateral, $\pm 5 \times 10^6$; for med nasal, $\pm 5 \times 10^6$.

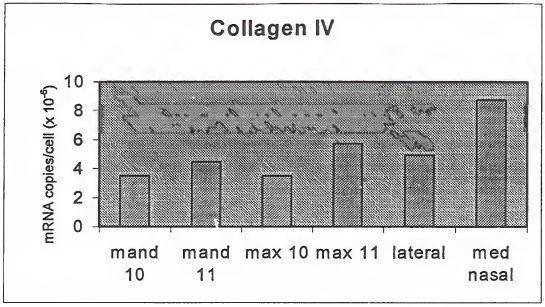


Fig.6. Steady state levels of mRNA copies per cell of facial processes for ECM genes measured by Q-RT-PCR for Collagen IV. Only a small increase in the steady state levels of collagen IV was observed. All reactions were made on three separate preparations of total RNA. Values are expressed as means of three determinations ± SEM. SEM values for mand 10, $\pm 2.1 \times 10^{5}$; for mand 11, $\pm 1 \times 10^{5}$; for max 10, $\pm 2 \times 10^{6}$; for max 11, $\pm 3.1 \times 10^{6}$; for lateral, $\pm 1.1 \times 10^{6}$; for med nasal, $\pm 1 \times 10^{6}$.

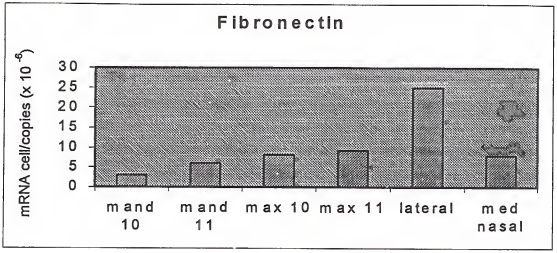


Fig.7. Steady state levels of mRNA copies per cell of facial processes for ECM genes measured by Q-RT-PCR for Fibronectin. Only a small increase in the steady state levels of fibronectin was observed. All reactions were made on three separate preparations of total RNA. Values are expressed as means of three determinations ± SEM. SEM values for mand 10, $\pm 2 \times 10^7$; for mand 11, $\pm 2 \times 10^7$; for max 10, $\pm 2.7 \times 10^7$; for max 11, $\pm 8 \times 10^7$; for lateral, $\pm 2.7 \times 10^7$; for med nasal, $\pm 6.5 \times 10^7$.

### Matrix Metalloproteinases and Tissue Inhibitors Expression Do Not Show Major Temporal Regulation During Facial Morphogenesis *In Vivo*

Zymograms of gelatin gels showed that all facial processes isolated from the three different gestational ages (10, 10.5 and 11 days) demonstrated a major 58 kDa band and minor bands at 50, 60, 90 and 100 kDa. These appear to be constitutively expressed. An additional minor band at 55 kDa was not seen in gestational day 10 facial processes, but appeared in all tissues from day 11. Bands at molecular weights of 50, 58 and 60 kDa had increased protease activity by 1.6 fold as development progressed (from day 10 to day 11), as indicated by densitometric analysis. Other minor bands appeared in tissues of day 11 at molecular weights of 28, 55/52, 72 and 120 kDa (fig. 8).

All gelatinases were inhibited by incubating samples with 4 mM EDTA or 1,10-phenanthroline prior to electrophoresis, demonstrating that gelatinolytic activity was due to matrix metalloproteinase activity. Twenty mM of serine protease inhibitor, phenylmethylsulfonyl fluoride (PMSF), did not affect gelatinolytic activity when incubated with samples prior to electrophoresis.

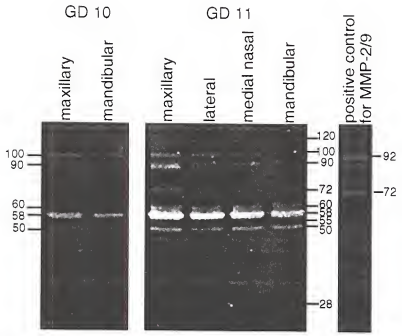


Fig.8. Matrix metalloproteinases in facial processes of gestational days 10 and 11. Zymography of gelatin gels with facial processes revealed proteolytic bands with major activity at 58 kDa and minor bands at 50, 60, 90 and 100 kDa. These appear to be constitutively expressed. An additional minor band at 55 kDa was not seen in gestational day 10 facial processes, but appeared in all tissues from day 11. Bands at molecular weights of 50, 58 and 60 kDa had increased protease activity by 1.6 fold as development progressed (from day 10 to day 11), as indicated by densitometric analysis. Other minor bands appeared in tissues of day 11 at molecular weights of 28, 55/52, 72 and 120 kDa Purified MMP-2 and MMP-9 (Chemicon) were used as positive controls and co-migrated with the samples. A 28 kDa band was only seen on day 11 samples. The molecular weight (kDa) is indicated in the side of the panel.

The zymographic results for gelatin gels are summarized in Tables 4 and 5.

**Table 4:** Summary of zymographic results for gelatin gels.

| | Day 10 | | Day 10.5 | | | Day 11 | | | |
|---|---|---|---|---|---|---|---|---|---|
| Active bands (kDa) | process Maxillary | process Mandibular | process Maxillary | process Mandibular | process Lateral | process Maxillary | process Mandibular | process Lateral | process Medial Nasal |
| 120 | - | - | - | - | - | + | + | + | + |
| 100 | + | + | + | + | + | + | + | + | + |
| 90 | + | + | + | + | + | ++ | + | + | + |
| 72 | - | - | - | - | - | + | - | - | - |
| 60 | ++ | + | ++ | + | + | ++ | ++ | ++ | ++ |
| 58 | +++ | ++ | +++ | +++ | +++ | ++++ | ++++ | ++++ | ++++ |
| 55/52 | - | - | - | - | - | + | + | + | + |
| 50 | + | + | + | + | + | ++ | ++ | ++ | ++ |
| 28 | - | - | - | - | - | + | + | + | + |

Varying degrees of activity: + = faint band, ++ = visible activity, +++ = activity, ++++ = very strong activity.

Four-aminophenylmercuric acetate (APMA) was used for activation of gelatinases. On gelatin gels, an active band at 120 kDa that appeared in all processes of day 11 could not be detected after activation with APMA. Also, a 55 kDa band had the most gelatinolytic activity with minor bands at approximately 58 kDa. Gelatinases activated

with APMA (Fig. 8) had a greater increase in gelatinolytic activity than the non-activated bands as detected by densitometric analysis, indicating organomercuric activation.

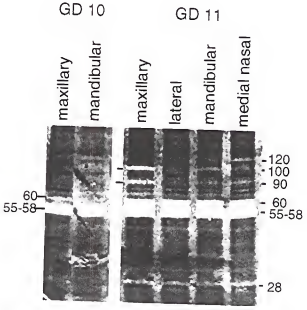


Fig.9. Matrix metalloproteinases in facial processes of gestational days 10 and 11. Zymography of gelatin gels with facial processes activated with APMA revealed proteolytic bands with major activity at 55 to 58 kDa range. The molecular weight (kDa) is indicated in the side of the panel.

**Table 5:** Summary of zymographic results for APMA activated samples on gelatin gels.

| | Day 10 | | Day 11 | | | |
|---|---|---|---|---|---|---|
| Active bands (kDa) | process Maxillary | process Mandibular | process Maxillary | process Mandibular | process Lateral | process Medial Nasal |
| 100 | - | - | ++ | + | - | + |
| 90 | - | - | ++ | + | + | + |
| 70/72 | - | - | - | - | - | - |
| 60 | - | - | + | + | + | + |
| 58 | + | + | ++ | ++ | ++ | ++ |
| 55 | ++ | ++ | +++ | +++ | +++ | +++ |
| 28 | - | - | + | + | + | + |

Varying degrees of activity: + = faint band, ++ = visible activity, +++ = activity, ++++ = very strong activity.

Zymography on casein gels did not demonstrate any bands using the procedures described above, but revealed a faint 28 kDa active band when using the Novex system (San Diego, CA) which utilizes a 4-16% Tris-Glycine gel with blue-stained beta casein incorporated as substrate. However, casein-plasminogen gels revealed proteinase bands with molecular weights at 54 kDa and at 40/38 kDa (Fig. 10). No difference in activity was observed between different ages. All plasminogen dependent caseinases were inhibited by 1 μg/ml of aprotinin, a specific inhibitor of serine proteases, added to samples and incubated at 37°C for 30 min prior to incubation.

Reverse zymograms were performed using collected conditioned media at 23.5% (vol) in 10% SDS-PAGE gels. All tissues demonstrated a 28 kDa band compatible with TIMP-1 with multiple high molecular weight bands suggesting inhibitor bound to proteases (Fig 11).

To further identify the MMPs present in the facial processes, Western blots were performed (Fig 12). With antibody to MMP-2 (IgG-antipeptide antibody), a major band at 58 kDa was detected. The molecular weight of this band corresponds with the most active degradation bands from the zymograms. Although this band is immunostained with MMP-2 antibody, its identity remains to be elucidated. The results here indicate it to be a differentially glycosylated form of the activated enzyme, a degraded form of MMP-2 or it might be a distinct enzyme altogether. Other bands are seen in the blot at different molecular weights (28 kDa, 80 kDa and 100 kDa) representing active degradation products at lower molecular weights and differentially glycosylated forms at higher molecular weights. The 58 kDa band was not stained by any other antibodies (i.e. MMP-3 and MMP-9) tested by Western immunoblotting. Similarly, controls for antibody specificity comprised of no incubation with the primary antibody demonstrated no bands.

Western blots for MMP-9 demonstrated multiple bands with the major reactivity at 90 kDa. Additional bands represent variants in glycosylation of the enzyme. Human placenta and purified human MMP-2 and MMP-9 (Chemicon, Temecula, CA) were used as positive controls for each blot. Negative controls were performed with secondary antibody only. Immunoblots probed with antibody to MMP-3 demonstrated the presence of a major band at 50 kDa.

Reverse transcriptase-polymerase chain reaction (RT-PCR) results indicated the presence of MMP-2, MMP-3, MMP-7 and MMP-9 mRNA but not MMP-1. All primers have been tested against other tissues such as salivary glands, which demonstrate their specificity, and through DNA sequence analysis of amplicon products. The identity of the PCR products was confirmed by using the Southern blot technique, which demonstrated the presence of the authentic amplicons for matrix metalloproteinases, MMP-2, MMP-3, MMP-7, MMP-9, the natural inhibitors, TIMP-1 and TIMP-2, and the their activators, uPA and tPA. These appear to be constitutively expressed since no major differences in band intensities were found between the different gestational days relative to G3PDH or β-actin expression using densitometric analysis (fig.13). An example of a RT-PCR reaction in an agarose gel and its corresponding southern blot is shown in Fig. 14.

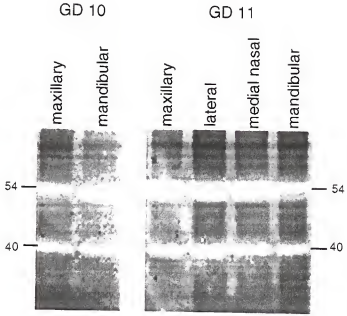


Fig.10. Presence of Serine proteases in facial processes of gestational days 10 and 11. Zymography of casein-plasminogen gels with facial processes revealed proteolytic bands with major activity at 54 and 40 kDa. These bands appeared to have same level of activity. The molecular weight (kDa) is indicated in the side of the panel.

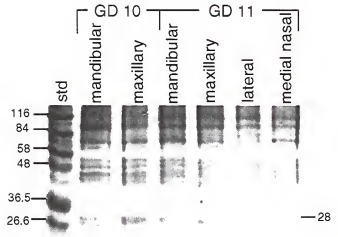


Fig.11. Reverse zymography was performed using collected conditioned media at 23.5% (vol) in 10% SDS-PAGE gels. All tissues demonstrated a 28 kDa band compatible with TIMP-1. The molecular weight (kDa) is indicated in the side of the panel.

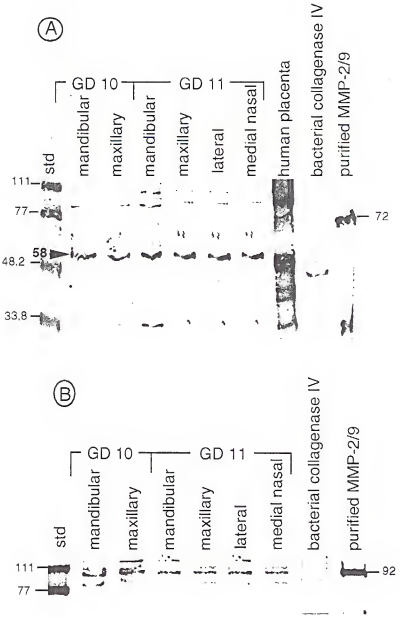


Fig.12. Presence of MMPs in the facial processes at the time of facial development. a) immunoblots for MMP-2 revealed a major band at 58 kDa. This corresponds with the most active degradation bands from the zymograms. Additional bands represent active degraded forms of the enzyme at lower molecular weights and differentially glycosylated forms at higher molecular weights. Purified MMP-2 (Chemicon) and human placenta were used as positive controls, and bacterial collagenase IV as negative control. b) western blots for MMP-9 demonstrated multiple bands with the major reactivity at 90 kDa. Additional bands represent variants in glycosylation of the enzyme. Human placenta and purified human MMP-9 (Chemicon, Temecula, CA) were used as positive controls. Molecular weight standards are: 111 kDa, Phosphorylase B; 77 kDa, Bovine serum albumin; 48.2 kDa, Ovalbumin; 33.8 kDa, Carbonic anhydrase.

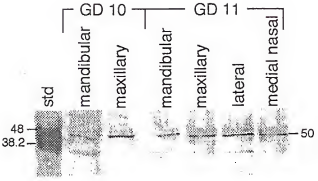


Fig.12cont. c) immunoblots probed with antibody to MMP-3 demonstrated the presence of a major band at 50 kDa. The molecular weight (kDa) is indicated in the side of the panel. Negative controls were performed with secondary antibody only (data not shown).

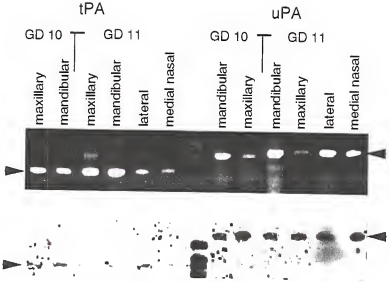


Fig.13. Expression of MMPs, TIMPs and PAs in the facial processes at the time of facial formation. Example of RT-PCR reaction with corresponding southern blot.

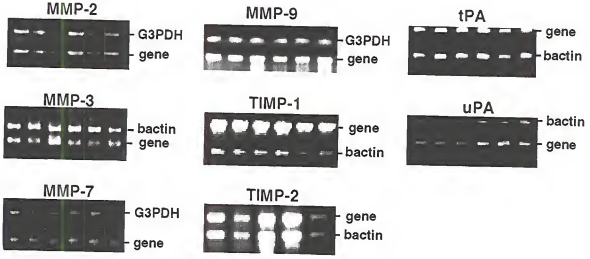


Fig.14. Representative composite data for RT-PCR. Results demonstrate the presence of MMP-2, MMP-3, MMP-7, MMP-9, TIMP-1, TIMP-2, uPA and tPA but not MMP-1 message. These appear to be constitutively expressed since no differences between the different gestational days to internal controls (β-actin or G3PDH) were found after densitometric analysis.

**Inhibition of Matrix Metalloproteinases Alters Facial Development *In Vitro***

In order to determine the role of MMPs in facial morphogenesis, a number of known MMP inhibitors were used to block proteinase function during in vitro development of mouse embryonic heads.

In normal facial development, the lateral and medial nasal processes on both sides of the developing nose make contact with each other and with their respective maxillary process at gestational day 11. Epithelial adhesion and later fusion occur at the inferior part of the nasal groove. Subsequently, this nasal fin disappears as adhered epithelial cells of the apposed lateral and medial nasal processes die or transdifferentiate into mesenchymal cells, basal lamina is degraded and a mesenchymal bridge between the processes that allows tissue continuity is formed (Diewert and Shiota, 1990). At mouse gestational day 13, the nose and upper lip are completely formed.

*In vitro* culture embryos treated with galardin, exhibited susbtantial deficiencies in morphogenesis. Generally, the embryonic heads had the same size, suggesting normal growth with defective or delayed differentiation. Inhibition was apparent at a range of concentrations beginning with 15 μg/ml of galardin, the lowest dose, as well as at 1 mg/ml, the highest dose. Galardin has been used in different systems as an inhibitor of MMPs and has been demonstrated to have minimal toxic effects (Schultz et al., 1992; Broverman et al., 1998).

Effects were also detected when a single dose of 150 μg/ml of galardin was administered to the cultured media for the first three days of culture only. Galardin or phenanthroline (10mM) specifically inhibited the fusion of lateral and medial nasal

processes, although the maxillary process appears to fuse to the lateral process. At gestational days 10+7 (7 days in culture), controls demonstrated complete facial formation and closure whereas explants cultured with galardin or phenanthroline showed a noticeable cleft resulting from the non fused lateral and medial nasal processes (Fig.15). Nonetheless, mandibular development did not seem affected, although it was delayed.

Histological examination revealed that lateral and medial processes remained close but did not fuse maintaining their structure with mesenchymal tissue encased by a layer of epithelial cells (Fig.16).

In contrast, embryo heads cultured with EDTA (1mM and 400 mM) exhibited gross morphological changes. The tissues displayed a gelatinous and dispersed organization that worsened as the time in culture progressed, culminating in total disarray of the head form.

Furthermore, embryo heads cultured in the presence of aprotinin (1.5μg/ml and 3.0μg/ml), a specific serine protease inhibitor, did not exhibit any morphological changes.

To further explore the role of MMPs activity and to identify the major proteinases of facial morphogenesis, a series of different concentrations of TIMP-2 (0.02 μg/ml, 0.05 μg/ml, 0.1 μg/ml, 0.2 μg/ml, 0.5 μg/ml and 1 μg/ml) (Albini et al, 1991), a natural inhibitor of MMP-2, were added to the media culture as described before. Although the embryo heads displayed features corresponding to arrested development, there were no apparent effects on nose development. Overall, the heads were small, approximately 50% of the controls size, and had a ball like shape, unlike the controls. The brain and the

forehead were particularly smaller in size compared to controls and to other inhibitor experiments, as were the lateral and medial nasal processes (Fig.17).

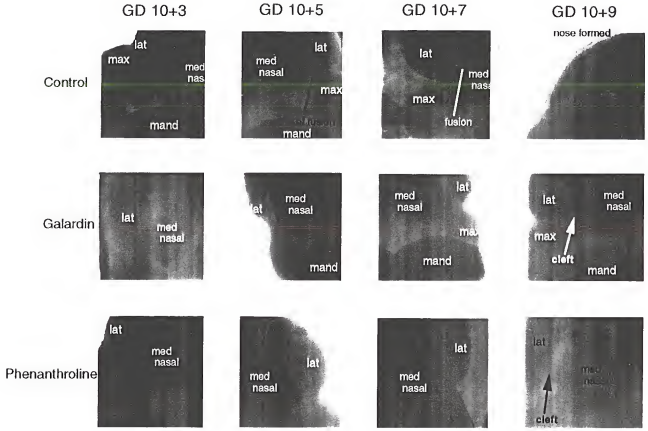


Fig.15. Use of MMP inhibitors in mouse embryonic head culture affected facial morphonesis. Galardin (15 μg/ml, 150 μg/ml and 1mg/ml) or phenanthroline (150 μl of solutions of 400mM) specifically inhibited the fusion of lateral and medial nasal processes. At gestational days 10+7 (7 days in culture), controls demonstrated complete facial formation and closure whereas experiments with galardin or phenanthroline showed a noticeable cleft resulting from the non fused lateral and medial nasal processes. All experiments were performed at least three times on separate occasions with five embryonic heads of gestational ages 10 or 10.5 for each treatment. Mand (mandibular process), max (maxillary process), lat (lateral process), med nasal (medial nasal process).

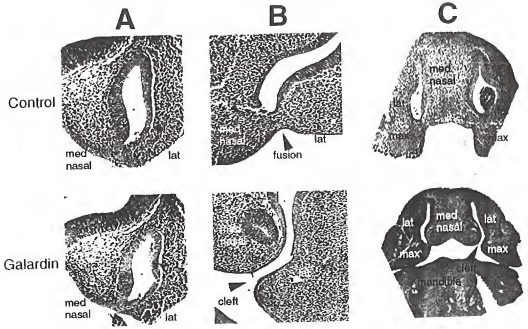


Fig.16. Hematoxylin-eosin stained frontal sections of mouse head embryos demonstrating the effect of MMP inhibitors on facial development: A) growth of lateral and medial nasal processes prior to fusion; B) controls demonstrated complete fusion after 9 days of culture whereas explants using galardin showed a cleft with defined separation of the processes. Epithelial layer and mesenchyme are noticeable at both sides of the cleft; C) lower magnification of fusion sites. Mand, mandibular process; max, maxillary process; lat, lateral process; med nasal, medial nasal process.

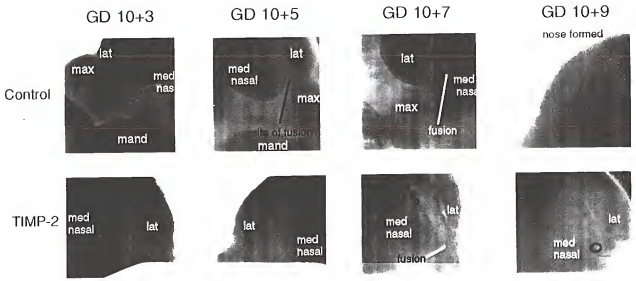


Fig.17. Effects of excess of TIMP-2 to culture of mouse embryo heads. The heads displayed features corresponding to arrested development but there were no apparent effects on nose development. Compared to the controls, experiment mouse embryo heads with TIMP-2 excess were small and had a ball like shape. A reduction of approximately 50% in size was observed. The brain and the forehead were particularly affected compared to controls and to other inhibitor experiments, as were the lateral and medial nasal processes. Dosage used was 0.02 μg/ml, 0.05 μg/ml, 0.1 μg/ml, 0.2 μg/ml, 0.5 μg/ml and 1 μg/ml. Each dose was used for at least 5 different embryo heads and repeated 3 times at different occasions.

# DISCUSSION

This study shows that a number of matrix metalloproteinases, their inhibitors and activators are expressed and present at the time of murine facial morphogenesis. Matrix metalloproteinases promote the degradation of extracellular matrix necessary for fusion of the facial processes. Normal growth and development of facial processes into mid-face and upper lip require cell proliferation and migration influenced by cell-extracellular matrix interactions and by epithelial-mesenchymal interactions regulated by signals that cross the basement membrane. Facial development also requires ECM synthesis and degradation performed by MMPs and mediated by the presence of inhibitors and by MMP activation.

The basement membranes of the lateral and medial nasal processes are degraded at the sites of fusion and replaced by a continuous mesenchymal tissue. Alterations of cell proliferation, cell migration or death, expression of certain genes such as homeobox genes, growth factors or ECM components may contribute to the appearance of cleft lip and palate. The results of this study suggest that facial morphogenesis is, in part, dependent on MMPs activity.

## Expression and Degradation of ECM Components by MMPs in Developing Facial Processes

Development and growth of the facial processes involve expression of extracellular matrix molecules. The distribution of basement membrane components in the mouse primary palate at the time of fusion has been reported recently (Iamaroon and Diewert, 1996). It has been demonstrated that a rapid degradation of the facial processes basement membrane and its components, laminin, collagen type IV and fibronectin, occurs at the time of completion of facial morphogenesis (Iamaroon and Diewert, 1996). Our investigation shows the presence of the same ECM components in the developing murine facial processes. The detectable amounts of collagen IV are decreased in maxillary, lateral and medial nasal processes of gestational day 11 compared to gestational day 10, suggesting degradation of basement membrane of these processes prior to fusion.

Quantitative RT-PCR data demonstrated that an increase in steady state mRNA levels of ECM genes occurs during facial development. The number of mRNA copies per cell obtained within the facial processes are similar to those of other studies using the same technique, Q-RT-PCR, but in different tissues (Macauley et al., 1997; Shim et al., 1997). Facial morphogenesis in mice occurs over the course of 3 days, therefore, the need for an increased production of structural proteins such as collagen III from gestational day 10 to 11 is not unexpected. At the same time, the increased steady state levels of mRNA copies per cell for collagen IV and fibronectin was much smaller. These two genes, along with laminin, have been found primarily in the basement membrane of facial processes (Iamaroon and Diewert, 1996), while fibronectin can also be found in the

mesenchyme. Since basement membrane is being degraded prior to fusion, we can speculate that there is little need for more of these molecules. However, laminin B1 is being produced in high numbers and an increase of its expression would be expected during highly proliferative stages due to its suggested roles in development. Laminin has additionally been implicated in cell migration, in regulating gene expression (Streuli et al., 1991) and has growth-like properties (Adams and Watts, 1993), therefore a modulation of its steady state concentration of mRNA potentially implies laminin as having a regulatory role in facial development.

Although the levels of ECM mRNA increased, MMP message steady state concentrations appear to remain constant during facial formation. Expression of MMPs and TIMPs have been investigated in many developing processes, including the craniofacial complex (Chin and Werb, 1997; Iamaroon et al., 1996; Werb, 1997). Diffuse labelling of matrix metalloproteinase-9 message was found by *in situ* hybridization in mandibular processes from gestational days 10 to 13 when it becomes more intensified in the mesenchyme surrounding the tooth buds (Chin and Werb, 1997). The same study demonstrated that MMP-3 was diffusely expressed in gestational days 9 to 15 mandibular processes. They also reported increased amounts of mRNA for MMP-2 as detected by RT-PCR. This study used RT-PCR methods where reactions were normalized to equal amounts of input total RNA and did not have an internal control. We report otherwise, according to our results, the message levels remained virtually constant as detected by RT-PCR where reactions were performed with same amounts of input total mRNA and were compared to positive controls (G3PDH or β actin) in the same reaction. Other studies have demonstrated the presence of MMP-2 in regions of the nasal and facial

prominences, with higher intensity in the zones of fusion of the lateral, medial nasal and maxillary processes (Iamaroon et al., 1996). In our studies, we not only detected mRNA for MMP-2, but also for MMP-3, MMP-7 and MMP-9. Zymographic evidence and Western blotting demonstrated that the proteolytic activity was possibly due to MMP-2, MMP-3 and MMP-9. Although the 58 kDa band was only immunostained with MMP-2 antibody, our results were inconclusive and we are uncertain of its identity. We can speculate of it being a differentially glycosylated form of the activated MMP-2 enzyme, a degraded form of MMP-2 or it might be a distinct enzyme altogether. Furthermore, the identity of the protease band relative to MMP-7 (28kDa) could not be confirmed due to the lack of antibodies.

Although some temporal differences in the MMPs appearance were found in the zymographic study, these differences were not reproduced in the Western blotting or the RT-PCR studies. It is likely that these differences reflect differential activation of MMPs, and differential activation and/or inhibition may be the modes of regulation of ECM degradation in facial morphogenesis. Although MMP-2 appears to be constitutively expressed during embryogenesis, there is accumulating evidence that other MMPs are expressed only when their activity is required. Our RT-PCR results demonstrate otherwise. Further work, probably using *in situ* hybridization, may elucidate these questions.

Matrix metalloproteinase-1 message was not found to be expressed during facial development. At the same time, collagen I mRNA was not detected in the facial processes by Q-RT-PCR. The major ECM substrates for MMP-1 are the interstitial collagens, among them, collagen type I. Considering the relation between enzyme and

substrate, we can speculate that with the absence of collagen I, there is no need for the expression of MMP-1. Many studies have shown that MMP-1 is not expressed until gestational day 14.5 or 15 in mouse embryos (Gack et al., 1995; Mattot et al., 1995). Its expression can be correlated with the onset of bone or cartilage formation.

**Matrix Metalloproteinase Degradation of the Basement Membrane Is Necessary for the Fusion of the Facial Processes**

Since we did not detect major temporal changes in the MMPs activity of facial processes, the question became how essential are these enzymes for proper craniofacial development. Correct facial morphogenesis relies on sufficient growth of the facial processes with subsequent fusion in a time dependent manner. The effects of MMP inhibitors on cultured mouse embryo heads indicate that, with inhibition of enzyme activity, fusion of lateral and medial nasal processes was retarded. However, overall cranial growth remained unaffected. The normal fusion of these processes requires degradation of basement membrane ECM molecules by allowing tissue continuity after cell migration. These migrations consequently result in upper lip and mid-face formation. Galardin specifically inhibited the fusion of lateral and medial nasal processes, whereas inhibitors for serine proteases did not exhibited the same effect, suggesting that MMPs are involved directly in this occurrence. The cleft resulting from the blocked fusion between the two processes has the basic characteristics of cleft lip (unilateral or bilateral), a common congenital defect. This data suggests that degradation of ECM by MMPs is a continuous process in facial development of the mouse. The observations here also indicate that MMP activity is necessary for proper formation of the mid-face.

Furthermore, genetic or environmental perturbations of these enzymes may contribute to the observed birth defect, cleft lip/palate.

Inhibition of facial morphogenesis, however, was not achieved by blocking MMP activity with TIMP-2. Since we detected the presence of MMP-2 and because of its suggested developmental role in other studies and its ability to degrade many ECM substrates, we initially hypothesized its having a major role in facial development. Because of TIMP-2's known inhibitory properties against MMP-2, by comparing both phenotypic results we could correlate whether the inhibition effects seen with galardin were also due to inhibition of MMP-2 activity. The results obtained here were inconclusive. Although TIMP-2 excess clearly affected overall cranial development reducing the size of the explants by approximately 50%, the nose seemed to develop normally. Some studies have suggested that it is necessary to achieve >90% inhibition of MMP activity to see morphological effect (Chin and Werb, 1997). At this gestational time point, the amount of MMP-2 in the facial processes is not known. Furthermore, the role of TIMP-2 and its interaction with MMP-2 are still being investigated. Recent reports have demonstrated that TIMP-2 inhibits the activation of pro-MMP-2 while others suggest that it may facilitate its activation through the formation of a complex with MT1-MMP (MMP-14) on the cell surface or to another TIMP-2 acting as a receptor. Recent reports of a TIMP-2 targeted mutation mouse suggest that TIMP-2 is necessary for the activation of MMP-2 (Caterina et al., 1998). Homozygous TIMP-2 knock-out mice only produced pro-MMP-2, whereas heterozygous produced both forms of the enzyme, pro and activated. It is also thought that MMP-2/TIMP-2 complex may still be

active but the level of gelatinase activity detected is only 10% of the free active MMP-2 (Kleiner et al., 1992; Yu et al., 1996).

It is interesting to speculate as to why many different types of matrix metalloproteinases (MMP-2, MMP-3, MMP-7 and MMP-9) with similar substrate specificities are expressed at the same time during morphogenesis. Results of transgenic mouse experiments with knock-outs for MMP-3, MMP-7, MMP-9, MMP-12 and TIMP-2 and overexpression of MMP-1 and MMP-3 were disappointing (Shapiro, 1997; Caterina et al., 1998). No dramatic phenotypes were produced in the analysis of embryonic developmental patterns. These results seem to give an impression of the redundancy of MMPs function. One or more enzymes may take over and compensate for each MMP knocked out. Potentially this is the case when TIMP-2 excess is used as an inhibitor of facial morphogenesis. However, when a broad spectrum inhibitor is used such as galardin, a variety MMP activity may potentially be blocked to some extent. The characterization of their genes have demonstrated that each enzyme may have a different control of expression (Huhtala, Chow and Tryggvason, 1990; Huhtala et al., 1991). By responding to different elements (for example, growth factors or the presence of inhibitors), the cell can regulate the expression of the different enzymes and change its surrounding environment.

It has been proposed that fusion of the hard palate is time-critical (Smiley, 1972). If the palatal shelves meet after the critical period for fusion, fusion will not take place. The same might also be true for facial morphogenesis, if the facial processes are not degraded in the critical time window, fusion will not occur. Therefore, the production of a high number of enzymes at the time of fusion is justified where one class of enzymes

can be substituted by another similar class but the absence or inhibition of a great number of different metalloproteinases can not be overcome. Perhaps, if degradation does not occur at the right time, other mechanisms such as apoptosis and cell migration may also be affected or prevented from occurring. This might explain why such a localized effect such as cleft lip and palate can occur in an otherwise healthy individual.

To date, detailed knowledge of how MMPs bind to their inhibitors and how the cell regulates the activation of MMPs remain to be clarified. Although a lot of work has been done in this field, MMPs are still being "discovered" (Pendás et al., 1997) which demonstrates how exciting and interesting it has been and yet has to come.

# CONCLUSIONS

This study indicates that MMPs are involved in facial morphogenesis by promoting migration of undifferentiated mesenchyme through degradation of the basement membrane prior to fusion of facial processes. A potential mechanism by which the mesenchymal bridge was prevented from occurring was by either inhibition of epithelial cell migration or apoptosis at the sites of fusion. The widespread expression and activity of MMPs suggest that other regulatory events may control local proteolysis therefore making the extracellular environment a dynamic one. We have developed a new approach that will be useful for investigating similar remodeling events *in vivo* or *in vitro*. The ability of *in vitro* culture experiments to generate a cleft lip phenotype identifies the matrix metalloproteinases as potentially a key component of proper facial morphogenesis. Fusion of the facial processes may also be time-critical. If degradation of extracellular matrix at the sites of fusion is inhibited for a period of time, fusion may not occur even at later stages. Such a critical event may require the activity of a large number of enzymes that may compensate for others if necessary.

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# BIOGRAPHICAL SKETCH

Adriana Costa Da Silveira was born in Rio de Janeiro, RJ, Brazil in September 30, 1968. She is the second of three children raised in Rio de Janeiro, RJ and Belo Horizonte, MG, Brazil. She received a D.D.S. degree from the Universidade do Estado do Rio de Janeiro in August 1990 and praticed general dentistry in Fortaleza, CE, Brazil for a year. There she met her husband and moved to Gainesville, FL, in 1991. Adriana began her graduate studies in the Department of Oral Biology in August 1993. She began working with Dr. Linda Brinkley in May 1994. She later transferred to Dr. Michael Humphreys-Beher's laboratory and he became her chair after Dr. Brinkley left the University of Florida.

The work in this study has been presented in several research meetings including the 25th American Association for Dental Research Meeting in San Francisco, CA, the 1997 American Edward H. Hatton Award at the 27th American Association for Dental Research Meeting in Minneapolis, MN, and the 1998 University of Florida Medical Guild competition. Dr. Da Silveira also received many awards and scholarships including the Colgate/Palmolive Hispanic Dental Association Scholarship, the Grinter Award from the Graduate School of the University of Florida and the Florida Brazil Institute.

Adriana began the Residency in Orthodontics program at the Eastman Dental Center/ University of Rochester in July 1998.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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